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14. ABSTRACT Variants of the contactin associated protein-like 2 (Cntnap2) gene are risk factors for language-related disorders including autism spectrum disorder, specific language impairment, and stuttering. Songbirds are useful models for study of human speech disorders due to their shared capacity for vocal learning, which relies on similar cortico-basal ganglia circuitry and genetic factors. We investigated Cntnap2 protein expression in the brain of the zebra finch, a songbird species in which males, but not females, learn their courtship songs. We hypothesized that Cntnap2 has overlapping functions in vocal learning species, and expected to find protein expression in song-related areas of the zebra finch brain. We found that Cntnap2 protein is enriched in several song control regions relative to surrounding tissues, particularly within the adult male, but not female, robust nucleus of the arcopallium (RA), a cortical song control region analogous to human layer 5 primary motor cortex. The onset of this sexually dimorphic expression coincides with the onset of sensorimotor learning in developing males. Enrichment in male RA appears due to expression in projection neurons within the nucleus, as well as to additional expression in nerve terminals of cortical projections to RA from the lateral magnocellular nucleus of the nidopallium. Cntnap2 protein expression in zebra finch brain supports the hypothesis that this molecule affects neural connectivity critical for vocal learning across taxonomic classes.

15. SUBJECT TERMS
Animal model, Autism susceptibility, Birdsong, Cntnap2, Communication, Social

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INTRODUCTION

The broad long-term objective of this proposal is to develop a novel model of autism spectrum disorder (ASD) using the zebra finch songbird. While no single model can capture all features of ASD, songbirds are one of the few in which the language subcomponent comprised by learned vocal communication can be studied. This is because songbirds, like humans but unlike traditional laboratory animals, learn a significant portion of their vocalizations (song) through social interactions with conspecifics. We previously showed that a key region of the brain song control system known as the robust nucleus of the arcopallium (RA) exhibits enhanced mRNA expression of the autism susceptibility gene contactin-associated protein-like 2 (*Cntnap2*) in male zebra finches (who learn to sing) at the onset of the sensorimotor phase of song learning (Panaitof et al., 2010). In contrast, in females of this species who do not learn to sing, *Cntnap2* levels decline in this same region at this time. This and other observations led us to hypothesize that Cntnap2 contributes to the regional and functional specification of brain regions important for socially-learned vocal communication in humans and songbirds, a key phenotype affected in ASD. In experiments designed to test this hypothesis, we have now shown that FoxP2 protein in RA follows the mRNA pattern, with a striking change between young male and female zebra finches between 35 and 50 days. During this time, Cntnap2 protein expression is enhanced in song-learning males at the onset of sensorimotor learning, whereas it declines in females. The enhancement occurs in RA projection neurons that are analogous to neurons in layer V primary motor cortex that, uniquely in vocal learners such as humans, make direct projections onto motor neurons controlling the muscles used in vocalization. This developmental switch within a privileged set of projection neurons provides the location and the timepoint at which to genetically intervene in male Cntnap2 expression and to determine the behavioral effects on song learning. Tests of this intervention are ongoing.

BODY

We proposed three aims to be completed across the three year funding period, as listed below. Here we provide a brief overview of our progress. During the first year, we completed all tasks enumerated under Aim 1 and made significant progress on Aim 2, task 5. Notably, we determined the developmental time frame at which Cntnap2 levels in RA diverge between male and female zebra finches. The major challenge we encountered in the second year was in identifying shRNA constructs that effectively knocked down zebra finch Cntnap2. The initial group of constructs proved ineffective when tested in human embryonic kidney (HEK) cells that were co-transfected with zebra finch Cntnap2. While we observed Cntnap2 overexpression in cells in which we provided the zebra finch Cntnap2 construct, there was no attenuation of this expression when we co-transfected shRNA constructs. We thus redesigned our shRNAs and obtained in vitro evidence that 3 were effective in knocking down zebra finch Cntnap2 in HEK cells, completing task 5 in Aim 2.

Another unexpected hurdle was our discovery that lentivirus was ineffective in driving suitable expression levels of a reporter gene in song control nuclei. After extensive testing of multiple viruses, we discovered an adeno-associated virus (AAV) serotype and promoter which effectively drive transgene expression in vivo and decided to use this instead of the lentiviral constructs previously proposed. This insight largely completed task 6 in Aim 2. With a suitable viral vector (AAV), promoter, and shRNA constructs in hand, in year 3 we began to test the knockdown efficacy of our constructs not just in HEK cells, but in primary neuronal cultures of zebra finch neurons. Moreover, we tested the efficacy of knockdown using single shRNAs versus combining them. Based on these experiments, we have ordered large scale production of an AAV construct from Virovek, Inc. and are currently awaiting its delivery. Outcomes thus far on each specific aim are enumerated below.

Aim 1: Determine the developmental expression pattern of CNTNAP2 protein in zebra finch brain (estimated to occur during years 1 and 2 of funding)

A manuscript describing the developmental and sexually dimorphic changes in Cntnap 2 protein levels in RA was published in 2014 (Condro & White, J Comp Neurol). Additionally, the PI wrote a review on songbirds as an animal model for human language disorders which was included in a book that was published in 2013.

Aim 2: Determine the effects of viral knock-down of CNTNAP2 in RA on songbird behavior (estimated to begin in year 1 and be completed in year 3)

We designed 4 non-overlapping short hairpin RNAs (shRNAs) against zebra finch Cntnap2. Plasmid constructs of these were prepared along with control constructs for nucleofection into cell cultures. Using both immortalized and primary neuronal cell culture systems, we performed extensive testing these constructs.

None were effective in knocking down zebra finch Cntnap2 relative to levels in cultures that received control constructs. Thus, in year 2 we redesigned six targeting constructs using new bioinformatic techniques (Task 5).
We have tested 3 of these, all of which substantially knock down exogenously expressed zebra finch Cntnap2 when co-transfected into HEK cells (Figure 1).

Knockdown levels achieved in HEK cells by single shRNAs ranged from 29% to 82% of control levels. Thus, to ensure robustness of the effect in vivo, we decided to test two shRNAs at a time, adding each as an independent construct, as well as to combine two shRNAs in one construct. We discovered that the latter was the most effective, achieving up to 100% knockdown in the cell lines (Table 1). We went on to test these combination plasmids in primary neuronal cultures made from zebra finch hatchling telencephala and to evaluate levels using two independent observers (Figure 2). We found up to 40% knockdown in this biologically more relevant system. We thus selected the dual construct for preparation of the virus.

Based on pilot studies using reporter genes, we determined that AAV achieves greater transgene expression in vivo than does lentivirus. We have thus now ordered AAV driving expression of the combo shRNA construct from Virovek. The company has experienced some delays in its preparation. We anticipate delivery in the coming weeks, after which we will proceed with in vivo stereotaxic injection and behavioral assessment, compared with the control viral construct.

Aim 3: Determine the effects of viral-mediated knock-down of *CNTNAP2* in RA on song circuit micro- and macro-connectivity. This aim awaits completion of Aim 2.

KEY RESEARCH ACCOMPLISHMENTS

Year One (Oct 2010 – 11)

Milestone 1: Animal approval.
Milestone 2: Identification of an antibody that specifically detects zebra finch Cntnap2 in brain tissue and cell culture.
Milestone 3: Detection of appropriate sized bands via Western analysis. Alternative validation was accomplished via exogenous expression of Cntnap2 in multiple cell lines.
Milestone 4: Collection of developmental series of male and female zebra finch brains.
Milestone 5: Identification of brain regions containing the key premotor song control nucleus, RA, as well as outlying cortical areas using Nissl staining.
Milestone 6: Determination of the age at which Cntnap2 protein becomes enriched in male RA and is diminished in female RA.
Milestone 7a: Partially complete. An abstract on this work was presented at the 2011 meeting of the Society for Neuroscience.

Year Two (Oct 2011 – 12)

Milestone 7b: We identified 3 shRNA constructs that effectively knockdown zebra finch Cntnap2 in vitro, and a control construct that does not affect these levels.
Milestone 8: Lentivirus was produced and delivered. However, it proved ineffective in driving robust transgene expression. This is not surprising given that since 2007, no publications have emerged that use lentivirus in songbirds to alter behavior. Thus, we switched to adeno-associated viruses for this goal.
Milestone 9: Partially complete: We identified tutors with complex songs and paired them with females for producing male sibling pairs.

Year Three (Oct 2012 – 13)

Milestone 7a: First research paper on this work is accepted for publication (Condro & White, J Comp Neurol, 2014a). Additionally, a book chapter describing this approach is published (White, 2013).
Milestone 7b: Decision to combine and test the 3 shRNA constructs in a pairwise fashion to determine whether greater knockdown efficacy can be achieved by expressing two targeting sequences in one vector. Testing reveals greater efficacy of one pair relative to single sequences.

Subsequent to Year Three (Aug 2013 – present)

Milestone 7a: An additional review paper is published (Condro & White, Comp Cog Behav Rev, 2014b)
Milestone 8: Adeno-associated virus is ordered from Virovek Inc.. Currently awaiting delivery.

REPORTABLE OUTCOMES

Published manuscripts

White SA (2013) Animal Models: Circuits and Molecular Networks for Vocal Learning, In: Origins of Language, edited by Claire Lefebre, Bernard Comrie & Henri Cohen, Cambridge University Press.

Condro MC & White SA (2014) Recent advances in the genetics of vocal learning. Comp Cog Behav Rev 9: 75-98.

Condro MC & White SA (2014) Distribution of language-related Cntnap2 protein in neural circuits critical for vocal learning. J Comp Neurol, 522: 169-185

Abstracts

Condro MC, Miller JE & White SA (2011) Autism susceptibility gene contactin associated protein-like 2 expression in a songbird model for vocal learning. Annual Meeting of the Society for Neuroscience, Washington DC, Abstract 150.19

Condro MC, Miller JE & White SA (2012) Autism susceptibility gene contactin associated protein-like 2 expression in a songbird model for vocal learning. Annual Retreat of the UCLA Interdepartmental Program in Molecular, Cellular & Integrative Physiology. *Poster received 'Best Student Presentation' prize.*

Condro MC, Miller JE & White SA (2012) Autism susceptibility gene contactin associated protein-like 2 expression in a songbird model for vocal learning. Dynamics of Neural Microcircuits Symposium, UCLA.

Presentations

Condro MC (2011) Can Cntnaps help zebra finches learn their song? Birdsong BRI Affinity Group Presentation, UCLA

Degrees

Michael C. Condro obtained his Ph.D. in Molecular, Cellular & Integrative Physiology in 2013.

Funding

Michael C. Condro obtained the 2012 Edith Hyde Fellowship awarded by UCLA's Department of Integrative Biology & Physiology.

Michael C. Condro obtained the 2011 Eureka Scholarship awarded by UCLA's Department of Integrative Biology & Physiology.

Employment

Dr. Michael C. Condro, Ph.D. obtained a postdoctoral scholar position in 2013.

Dr. Julie E. Miller, Ph.D. obtained a tenure-track faculty position in 2013 at the University of Arizona in the Departments of Neuroscience and of Speech, Language and Hearing Sciences.

CONCLUSION:

Our studies validate the use of the songbird model as a research organism relevant to understanding deficits in socially-learned vocal communication signals, such as those that are impaired in autistic individuals. As in humans, we found that the Cntnap2 autism susceptibility gene is enriched in brain areas that support learned vocal communication. We extended the known mRNA profile of expression to the protein level. One vocal control region of the songbird brain stood out as being particularly relevant to the human condition, namely the vocal control nucleus known as RA. This songbird brain region is analogous to primary motor cortex in humans, and its projection neurons, to layer V. In both songbirds and humans, but not in non-vocal learners, these projection neurons directly innervate the motor-neurons controlling phonation (e.g. syringeal and laryngeal motor neurons, respectively). We found that in zebra finches, as in humans, Cntnap2 protein is expressed in these neurons.

Interestingly, across the critical period of song learning in male zebra finches, Cntnap2 expression becomes enriched in these neurons, whereas it wanes in females who do not learn to sing. This suggests that Cntnap2 expression may be critical for this innervation, or in some other way support the sensorimotor learning process for vocalization. Our goal is thus to intervene in this expression using shRNA constructs designed to

knockdown Cntnap2 expression in vivo. In the process of our experiments, we learned a few lessons. The first was to design our own shRNA constructs based on relevant sequences in the songbird brain, rather than to utilize constructs designed for rodents. The second is to independently test viral constructs for their ability to express the relevant transgene in the specific neuronal population under investigation.

We are confident that we have learned these lessons and will soon be in receipt of an effective knockdown virus, which will enable us to pursue the outstanding questions from our proposal. These are the behavioral and, potentially based on our funding situation, the electrophysiological consequences of this intervention.

The impact of this work is to validate the songbird as an animal model that exhibits strengths over traditional lab animals such as rodents in determining the impact of genes on vocal learning behavior. The similarities in a key cell type (layer V primary motor cortex projection neurons in humans, and projection neurons of the robust nucleus of the arcopallium, aka RA, in the songbird) including that they both are enriched for Cntnap2 expression, provides a platform for testing the impact of attenuated Cntnap2 levels. A major mutation in Cntnap2 leads to language regression, seizures and other autistic symptoms in humans (Stephan, 2008). Disentangling each of these effects in the brain is a daunting task. By focusing on expression specifically within a vocal control nucleus, our work in songbirds promises to distinguish the impact of Cntnap2 specifically on vocal motor function in relation to more general impacts on brain development.

REFERENCES:

Condro MC & White SA (2014a) Distribution of language-related Cntnap2 protein in neural circuits critical for vocal learning. J Comp Neurol, 522: 169-185

Condro MC & White SA (2014b) Recent advances in the genetics of vocal learning. Comp Cog Behav Rev 9: 75-98.

Panaitof SC, Abrahams BS, Dong H, Geschwind DH & White SA (2010) Language-related *Cntnap2* is differentially expressed in sexually dimorphic nuclei essential for vocal learning in songbirds. J Comp Neurol, 518:1995-2018.

Stephan DA (2008) Unraveling autism. Am J Hum Genet, 82: 7-9.

White SA (2013) Animal Models: Circuits and Molecular Networks for Vocal Learning, In: Origins of Language, edited by Claire Lefebre, Bernard Comrie & Henri Cohen, Cambridge University Press.

APPENDICES: Please see attached papers at the end of this report: Condro & White, 2014 a, b; White, 2013

SUPPORTING DATA:
Figures

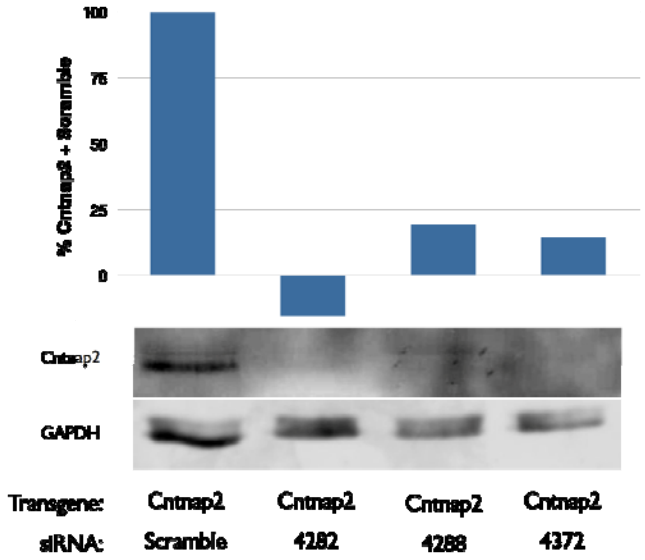

Figure 1. Testing shRNA constructs in HEK cells. siRNA constructs designed to target zebra finch Cntnap2 were coexpressed in HEK 293 cells along with the construct containing coding sequences for zebra finch Cntnap2. All 3 constructs were able to prevent >75% of Cntnap2 from being expressed relative to a non-targeting scrambled control.

Construct ID	Cells with knockdown	Total cells	% Knockdown	Observer 1	Observer 2	Recount
pCIG	20	257	7.8	20/258	19/256	--
shGEN	8	155	5.2	6/160	10/149	--
4282+4288	100	251	39.8	112/232	87/253	100/251
4328+4288	26	75	34.7	26/73	26/76	--
4328+4372	128	344	37.2	141/311	111/345	128/344

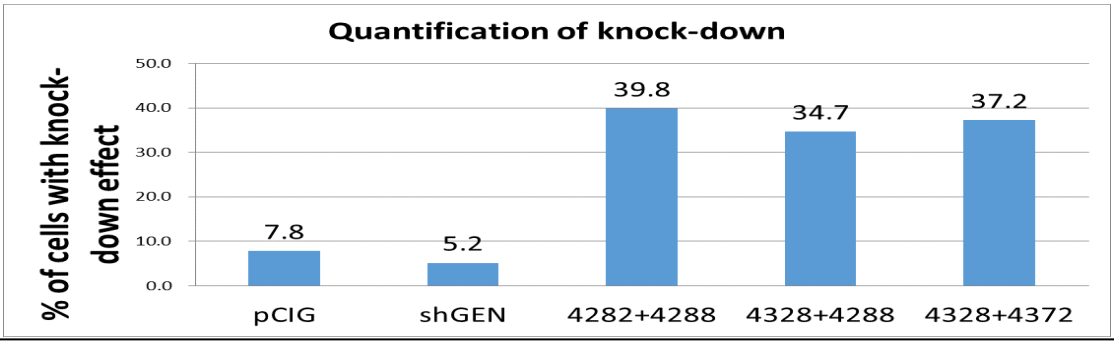

Figure 2. Effect of combo shRNA constructs in knocking down endogenous zebra finch Cntnap2 in primary telencephalic cultures shown in tabular (top) and graphic (bottom) forms. The two negative control constructs, pCIG and shGEN, do not substantially affect expression whereas each of the combination constructs achieves a ~35% reduction.

TABLE 1. Level of zebra finch Cntnap2 expression in HEK cells following transfection with a plasmid expressing zebra finch Cntnap2 plus either: another plasmid expressing one of the shRNA sequences (Single); two such plasmids (Double) or; a plasmid containing two shRNAs (Combo). Percentages are based on levels observed in HEK cells transfected with the zebra finch Cntnap2 expressing plasmid only.

Construct	shRNA ID	Cntnap2 Levels	% Knockdown
Single	4282	71%	29
	4288	41%	59
	4328	22%	78
	4372	18%	82
Double	4282, 4288	16%	84
	4328, 4372	25%	75
	4288, 4328	22%	78
Combination	4282+4288	3%	97
	4328+4372	2%	98
	4288+4328	0%	100

Distribution of Language-Related Cntnap2 Protein in Neural Circuits Critical for Vocal Learning

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ABSTRACT

Variants of the contactin associated protein-like 2 (Cntnap2) gene are risk factors for language-related disorders including autism spectrum disorder, specific language impairment, and stuttering. Songbirds are useful models for study of human speech disorders due to their shared capacity for vocal learning, which relies on similar cortico-basal ganglia circuitry and genetic factors. Here we investigate Cntnap2 protein expression in the brain of the zebra finch, a songbird species in which males, but not females, learn their courtship songs. We hypothesize that Cntnap2 has overlapping functions in vocal learning species, and expect to find protein expression in song-related areas of the zebra finch brain. We further expect that the distribution of this membrane-bound protein may not completely mirror its mRNA distribution due to the distinct subcellular localization of the two molecular species. We find that Cntnap2 protein is enriched in several song control regions relative to surrounding tissues, particularly within the adult male, but not female, robust nucleus of the arcopallium (RA), a cortical song control region analogous to human layer 5 primary motor cortex. The onset of this sexually dimorphic expression coincides with the onset of sensorimotor learning in developing males. Enrichment in male RA appears due to expression in projection neurons within the nucleus, as well as to additional expression in nerve terminals of cortical projections to RA from the lateral magnocellular nucleus of the nidopallium. Cntnap2 protein expression in zebra finch brain supports the hypothesis that this molecule affects neural connectivity critical for vocal learning across taxonomic classes. J. Comp. Neurol. 522:169–185, 2014.

INDEXING TERMS: autism; birdsong; Caspr2; speech; zebra finch

Language is a complex phenotype unique to humans, although facets of the behavior are shared with other species. Vocal learning, the ability to imitate or to produce novel sounds, is rare in the animal kingdom, so far found only in bats, cetaceans, elephants, pinnipeds, and songbirds. Humans are the only living primate species with this ability (Knornschild et al., 2010; Fitch, 2012; Stoeger et al., 2012). Genes underlying vocal learning and language are beginning to emerge, with a major breakthrough being the identification of Forkhead Box P2 (FOXP2) as the monogenetic locus for a human speech disorder. (Abbreviations in all capitals denote the human form of the molecule, lowercase is used for animal homologs, and italics denote nucleic acids.) FOXP2 is a transcription factor, and a mutation in its DNA binding domain leads to orofacial dyspraxia in a multigenerational pedigree known as the KE family (Lai et al., 2001). Additional FOXP2 mutations are associated with specific language impairment (SLI) and developmental verbal dyspraxia, further strengthening the link between the gene and language ability (Graham and Fisher, 2013). As a transcription factor, FOXP2's effects on language must be mediated through its gene targets. Chromatin immunoprecipitation has revealed that contactin associated protein-like 2 (*CNTNAP2*) is a direct transcriptional target of FOXP2 (Vernes et al.,

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2008). *CNTNAP2* is a particularly interesting target because it has independently been associated with a language-related disorder. Specifically, Old Order Amish children afflicted with cortical dysplasia-focal epilepsy (CDFE) harbor a deletion in *CNTNAP2*. CDFE is characterized by epilepsy, mental retardation, hyperactivity, impaired social behaviors, and language regression. A majority of affected children meet criteria for autism spectrum disorder (ASD), of which language impairment is a core deficit (Strauss et al., 2006). Within the general population, *CNTNAP2* polymorphisms are associated with language-related disorders, including increased risk for ASD (Arking et al., 2008; Li et al., 2010), delayed age of first word (Alarcón et al., 2008), SLI (Newbury et al., 2011; Peter et al., 2011; Whitehouse et al., 2011), and decreased long-range connectivity of the medial prefrontal cortex (Scott-Van Zeeland et al., 2010).

The mechanistic basis of these disorders is still unclear. The best characterized function of Cntnap2 is to cluster voltage-gated potassium channels (VGKCs) to the juxtaparanodes of nerves (Poliak et al., 2003; Horresh et al., 2008). In the central nervous system, Cntnap2 may also affect synaptic development (Anderson et al., 2012). Transgenic mice lacking *Cntnap2* exhibit behavioral abnormalities reminiscent of patients with CDFE, namely, epilepsy, hyperactivity, diminished social activity, repetitive behaviors, and reduced frequency of ultrasonic vocalizations when pups are separated from their dams (Peñagarikano et al., 2011). This diminished vocal behavior could be due to vocal impairment or lack of motivation as a form of reduced social activity. In either case, this aspect of the model is limited because pup isolation calls are innate. Songbirds, including zebra finches, offer an advantageous model for studying the impact of Cntnap2 given that they are vocal learners with a well-characterized neural circuitry that underlies this ability.

Like other songbirds, zebra finches possess a distinct set of interconnected brain nuclei dedicated to vocal learning and production termed the "song circuit" (Fig. 1). The circuit consists of two pathways: the posterior vocal pathway, required for vocal production, includes a projection from the cortical nucleus HVC (proper name; Reiner et al., 2004) to the robust nucleus of the arcopallium (RA), which in turn projects to the hypoglossal nucleus (nXIIts) that controls the avian vocal organ, the syrinx (Nottebohm et al., 1976). The anterior forebrain pathway (AFP), required for song modification (Brainard and Doupe, 2000), begins with a separate subset of HVC projections to the striatopallidal nucleus area X, which projects to the medial portion of the dorsolateral nucleus of the anterior thalamus (DLM), which then projects to the lateral magnocellular nucleus of the nidopallium (LMAN), which sends nerves terminals to RA as well as back to area X. This latter pathway is a cortical-basal ganglia-thalamo-cortical loop similar to the circuitry thought to underlie vocal learning in humans (Simonyan et al., 2012). An advantage of the zebra finch model is that vocal learning behavior and anatomy is sexually dimorphic. Females have an incomplete song circuit in which area X is not fully developed (Nottebohm et al., 1976), and RA is not innervated by HVC, causing the nucleus to shrink through apoptosis (Konishi and Akutagawa, 1985; Nixdorf-Bergweiler, 1996). Consequently, males begin to sing around 35 days (d) (Immelmann, 1969; Price, 1979), whereas females have never been observed to sing in nature. The sexually dimorphic singing behavior and the underlying song circuit anatomy make zebra finches an advantageous model for studying genes related to vocal learning including human speech.

As an initial step toward using songbirds as a model for vocal deficits associated with Cntnap2, Panaitof et al. (2010) described endogenous mRNA expression in the zebra finch. Remarkably, *Cntnap2* punctuates the song circuit with differential expression in song nuclei relative to their surrounding tissues. In juvenile and adult males, *Cntnap2* is enriched in two cortical song nuclei, RA and LMAN, but diminished in area X. In females, *Cntnap2* levels in RA and LMAN are equivalent

Abbreviations

AD	Dorsal arcopallium
AFP	Anterior forebrain pathway
AIV	Ventral intermediate arcopallium
Arco	Arcopallium
Cntnap2	Contactin associated protein-like 2
d	Days of age
DLM	Medial portion of the dorsolateral nucleus of the anterior thalamus
DMP	Dorsomedial nucleus of the posterior thalamus
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GP	Globus pallidus
Gran	Granule cell layer of the cerebellum
Hyper	Hyperpallium
Kvβ2	Potassium channel beta subunit
LFB	Lateral forebrain bundle
LMAN	Lateral magnocellular nucleus of the anterior nidopallium
Meso	Mesopallium
Mol	Molecular cell layer of the cerebellum
NeuN	Neuronal nuclei
Nido	Nidopallium
nXIIts	Hypoglossal nucleus
Ov	Ovoid nucleus
Pur	Purkinje cell layer of the cerebellum
PV	Parvalbumin
RA	Robust nucleus of the arcopallium
St-P	Striatopallidum
VGKC	Voltage-gated potassium channel
X	Area X
ZFTMA	Zebra finch immortalized cell line

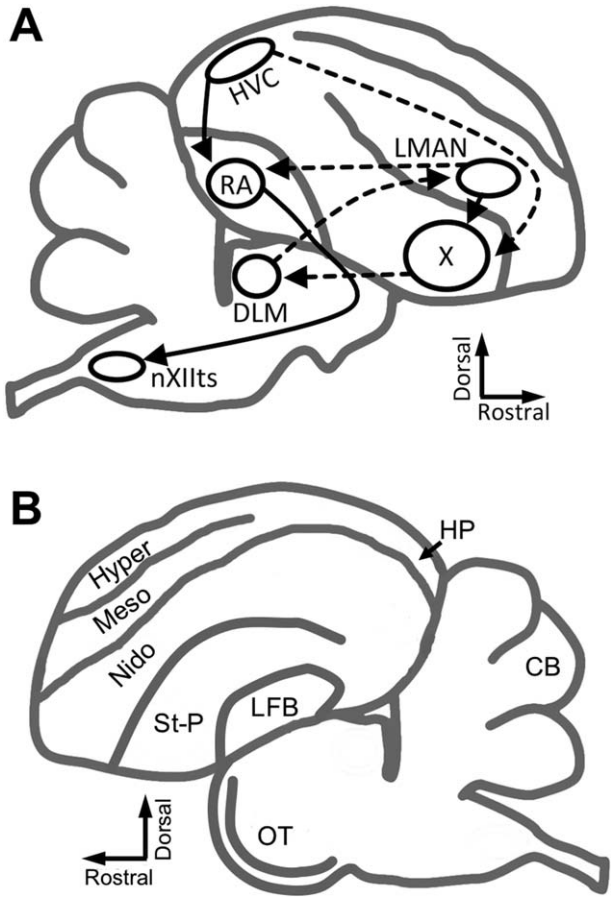

Figure 1. Diagram of the songbird brain. **A:** Schematic sagittal drawing depicts simplified song control circuitry. Solid lines indicate the posterior motor pathway, beginning with HVC, which projects to RA. RA directly projects to nXIIts, which controls the motor neurons of the syrinx. Dashed lines indicate connections of the AFP, in which HVC, X, DLM, and LMAN comprise a cortical-basal ganglia-thalamo-cortical loop like those underlying procedural learning in mammalian brains. LMAN completes the song circuit by projecting to RA, as well as back to X. **B:** Schematic sagittal drawing depicts nonsong brain regions in which Cntnap2 immunostaining was analyzed in this study. See list for abbreviations.

to or lower than those of the surrounding arco- and nidopallium, respectively (Panaitof et al., 2010). Differential *Cntnap2* expression in the song circuit suggests that it serves a purpose in vocal learning (White, 2010; Hilliard et al., 2012). If so, translation is required for any effects on anatomy or physiology. Protein expression does not always follow that of the encoding mRNA, with a precedent in songbirds for socially regulated translation (Whitney and Johnson, 2005). We hypothesized that protein expression patterns would be largely similar to those for the mRNA, but with some differences due to posttranscriptional changes and to localization of the protein not only to cell bodies, but also along axons.

Here we validate an antibody against Cntnap2 for use in zebra finch tissue and describe the Cntnap2 protein distribution in the zebra finch brain at timepoints during male song development. We find that expression in song circuit neuronal cell bodies largely follows the mRNA but also highlights axonal connections critical for the vocal learning capacity. In line with this idea, within the sexually dimorphic nucleus RA, we identify projection neurons as the cell type that expresses Cntnap2 protein.

MATERIALS AND METHODS

Animals and tissue preparation

All animal use and experimental procedures were in accordance with the National Institutes of Health (NIH) guidelines for experiments involving vertebrate animals and approved by the UCLA Chancellor's Animal Care and Use Committee. Zebra finches (n = 32 male and 21 female) between 25 and 500 days of age (d) used in this study were obtained from our breeding colony. Sex was determined based on sexually dimorphic plumage, or by postmortem identification of gonads at ages prior to the emergence of dimorphic plumage.

Antibody characterization

Cntnap2

In order to assess endogenous zebra finch Cntnap2 protein levels and distribution, a commercially available anti-Cntnap2 primary polyclonal antibody (Table 1) was selected for testing based on the perfect homology of the antigenic site (amino acids 1315–1331 in the C terminus of NCBI accession number NP_054860) between humans, rats, mice, and zebra finches. A translated nucleotide BLAST (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) search revealed no other plausible targets in the zebra finch genome. The ability of this antibody to detect zebra finch Cntnap2 was vetted as described below (Fig. 2).

Gapdh

Used here to measure relative levels of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) as a loading control in western analysis, the antibody (Table 1) detects a 38-kDa band in mammalian lysates, according to the manufacturer. It has been previously used in western analysis in mice (Jones et al., 2008; Fortune and Lurie 2009) and in zebra finch (Miller et al., 2008; Hilliard et al., 2012), detecting a protein band ~36 kDa in the latter animal.

Potassium channel beta subunit (Kvβ2)

The Kvβ2 antibody (Table 1) was selected for use in zebra finch due to perfect homology of the antigenic

TABLE 1.
Primary Antibodies

Primary antibody	Immunogen	Manufacturer	Catalog no.	Species
Cntnap2 (Caspr2)	Synthetic peptide corresponding to amino acids 1315–1331 of rat Caspr2, accession number NP_054860)	Millipore (Temecula, CA)	AB5886	Rabbit polyclonal
Gapdh	Purified GAPDH from rabbit muscle	Millipore	MAB374	Mouse monoclonal
Kvβ2	Amino acids 17–22 of rat Kvβ2 (accession number NP_034728), conserved in zebra finch	Neuromab (Davis, CA)	K17/70	Mouse monoclonal
NeuN	Purified cell culture nuclei from mouse brain	Millipore	MAB377	Mouse monoclonal
Parvalbumin	Parvalbumin purified from carp muscles	Swant (CH)	235	Mouse monoclonal

site, amino acids 17–22 (TGSPG) of rat (accession number NP_034728), and zebra finch (NCBI RefSeq NC_011485.1). A translated nucleotide BLAST search revealed no other plausible targets of the antibody in zebra finch. Specificity of this antibody is described by the manufacturer. In western analysis, the antibody detects a major protein band at 38 kDa and a minor band at 41 kDa in brain lysates from wildtype mice, but no bands in lysates from knockout mice (http://neuromab.ucdavis.edu/datasheet/K17_70.pdf). Although the specificity of this antibody has not been confirmed for use in zebra finch, a recent study using this antibody found significant overlap of Kv1.1, Kv1.2, and Kvβ2 (Ovsepian et al., 2013), suggesting that even if the antibody is not specific to Kvβ2, it will at least have a similar immunostaining pattern. We use this antibody only to show that Cntnap2 colocalizes with potassium channel subunits and do not make any claims as to its specificity.

NeuN

The anti-NeuN antibody (Table 1) was used in this study to identify morphology in the zebra finch brain, as it was in Scott and Lois (2007). According to the manufacturer, the antibody detects protein bands at 46 and 48 kDa in western analysis.

Parvalbumin

The anti-parvalbumin antibody (Table 1) was characterized in Celio et al. (1988). It has since been used to detect the zebra finch isoform in immunohistochemistry to identify parvalbumin-positive neurons in song control nuclei (Wild et al., 2001, 2005, 2009; Roberts et al., 2007), as it is used in this study.

Cell culture

Whole brain homogenate was obtained from an adult male zebra finch. Following overdose with inhalation anesthetic (isoflurane, Phoenix Pharmaceutical, St. Joseph, MO), the brain was dissected without fixation and homogenized with a hand-held homogenizer (Kontes, Thermo Fisher Scientific, Pittsburgh, PA) in ice-cold modified RIPA lysis buffer (pH 7.6) with protein inhibitors (No. P8340, Sigma-Aldrich, St. Louis, MO) and an RC DC Protein Assay (Bio-Rad, Hercules, CA) was performed to determine protein concentration as in Miller et al. (2008). Zebra finch ZFTMA cells (Itoh and Arnold, 2011) which do not endogenously express Cntnap2 (Fig. 2B) were transfected with either a pCR-TOPO vector (Life Technologies, Grand Island, NY) containing the complete coding sequence for zebra finch Cntnap2 (Panaitof et al., 2010) or a pGIPz vector (Thermo Scientific, Lafayette, CO) containing the GFP coding sequence only, as a negative control. Cells were transfected using a Nucleofector II and chicken nucleofector solution (Lonza, Basel, Switzerland) and distributed on BD Falcon tissue culture dishes (100 × 20 mm style, Fisher Scientific). At 24 hours posttransfection, GFP expression was observed in ~70% of cells in the plate transfected with the pGIPz vector (not shown). Forty-eight hours after transfection, cells were dissolved in ice-cold modified RIPA lysis buffer with protease inhibitors and a protein assay was performed as above.

Western analysis

Samples of both brain homogenates and cell culture lysates were prepared for immunoblotting by diluting with 2× 5% betamercaptoethanol in Laemmli buffer (pH 6.8; Bio-Rad) and storing at −80°C until use. Samples of 25 μg of brain and 100 μg of cell culture lysates were boiled for 2 minutes and then resolved on a 10% isocratic sodium dodecyl sulfate (SDS)-polyacrylamide gel in Tris-glycine-SDS buffer (pH 8.3; Bio-Rad) at 100 V. A Precision Plus Protein Dual Color Standard (Bio-Rad) was included on the gel as a molecular mass marker. Protein was then transferred onto a PVDF membrane with a pore size of 0.45 μm in Tris-glycine (Bio-Rad) with 20% methanol and 1% SDS. The membrane was blocked with 5% milk in Tris-buffered saline

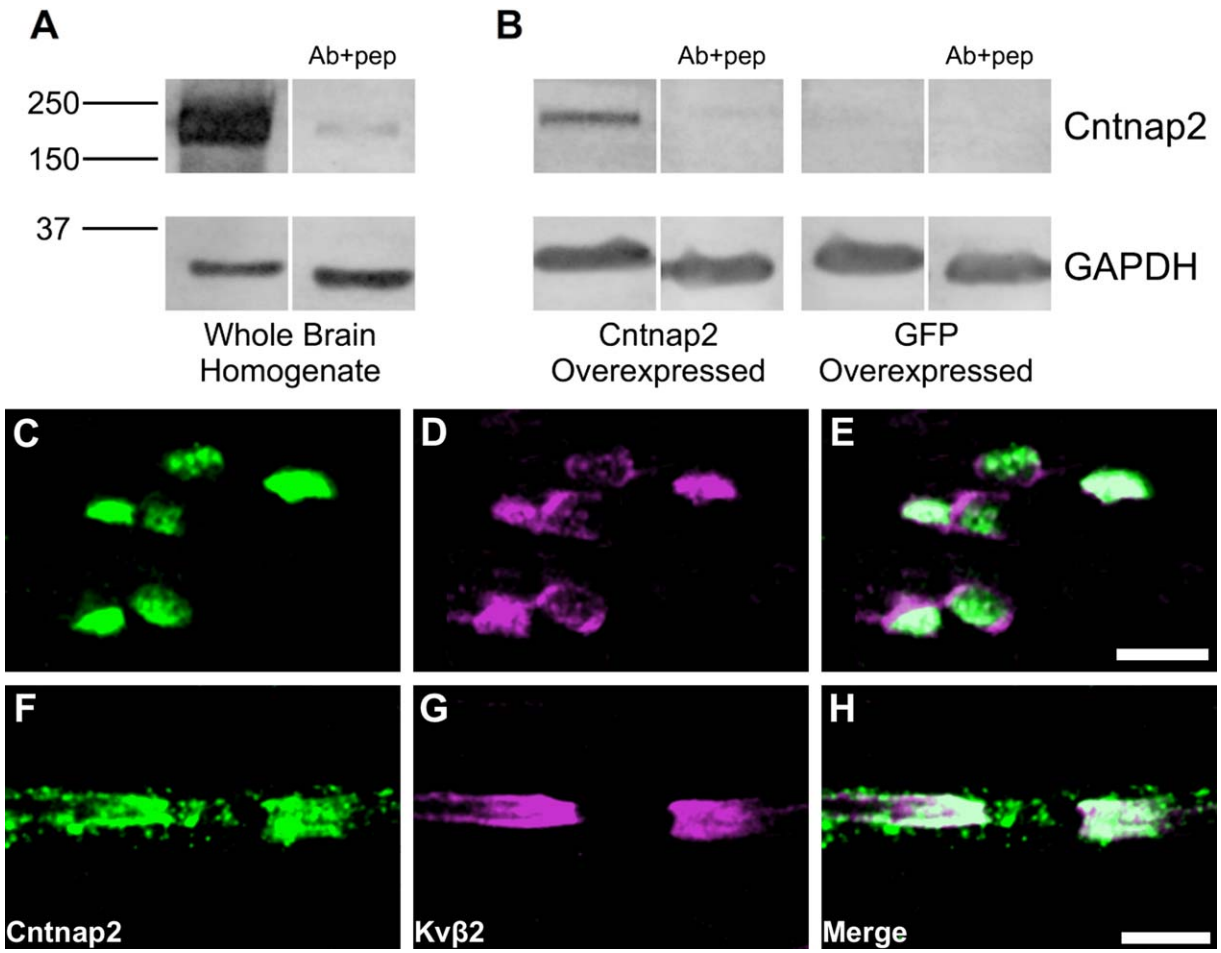

Figure 2. Antibody detection of zebra finch Cntnap2. **A:** Western blot of zebra finch whole brain homogenate. Anti-Cntnap2 primary antibody detects a single prominent protein band at the predicted molecular weight (~180 kDa) for endogenous zebra finch Cntnap2. **B:** Western blots of the ZFTMA zebra finch established cell line with a plasmid expressing zebra finch Cntnap2 or GFP. Transfection of the Cntnap2 construct results in a detectable signal at the predicted molecular weight for Cntnap2 (left). In contrast, transfection of GFP results in no detectable signal at the same molecular weight, confirming no endogenous Cntnap2 expression in this skin-derived cell line (right). For each condition, preadsorption of the primary antibody with its antigenic peptide (Ab+pep) dramatically reduces or removes the signal. Molecular weight markers are given in kDa. **C–E:** Zebra finch optic and **(F–H)** sciatic nerves double-labeled with Cntnap2 and potassium channel subunit Kvβ2 antibodies. Cntnap2 signal colocalizes with putative signals for potassium channel subunit Kvβ2 in both nerves, consistent with its expression in rodents (Poliak et al., 1999, 2003). Overlap of these signals in zebra finch nerves further validates the Cntnap2 antibody for use in this model. See list for abbreviations. Scale bars = 10 μm in C–E; 5 μm in F–H. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with 0.1% tween-20 (pH 7.4; TBST) for 2 hours and then incubated with the anti-Cntnap2 antibody at 1:250 and anti-Gapdh (Table 1) at 1:100,000 in 2.5% milk-TBST overnight at 4°C. A replicate set of samples was incubated with the anti-Cntnap2 antibody that had been preadsorbed with antigenic peptide (Millipore, Temecula, CA) at a ratio of 1:30 by mass. Blots were then incubated with horseradish peroxidase (HRP)-conjugated antirabbit and antimouse secondary antibodies (Table 2) at 1:2,000 and 1:10,000, respectively, in 2.5% milk-TBST for 2 hours. Blots were developed with ECL Plus, imaged on a Typhoon scanner (GE Healthcare), and signal specificity assessed.

Tissue staining and immunohistochemistry

Dissection and preparation of tissues

Birds of known age and sex were overdosed with isoflurane, then transcardially perfused with warmed saline followed by 4% paraformaldehyde in phosphate-buffered saline (pH 7.4; PBS). Brains were dissected out and cryoprotected in a 20% sucrose solution. Forty-μm thick sections were cut in either the coronal or sagittal orientation on a cryostat (Leica Microsystems, Bannockburn, IL) and thaw-mounted onto microscope slides (Colorfrost Plus; Fisher Scientific, Pittsburgh, PA) in a manner that produced replicate sets of adjacent or near-adjacent sections, then stored at −80°C until use.

TABLE 2.
Secondary Antibodies

Catalog no.	Manufacturer	Reactivity	Conjugate
NA931	GE Healthcare, Piscataway, NJ	Mouse IgG	Horseradish peroxidase (HRP)
NA934	GE Healthcare	Rabbit IgG	HRP
A-11008	Life Technologies, Grand Island, NY	Rabbit IgG	Alexa-Fluor 488
A-11001	Life Technologies	Mouse IgG	Alexa-Fluor 488
A-21422	Life Technologies	Mouse IgG	Alexa-Fluor 555
A-11004	Life Technologies	Mouse IgG	Alexa-Fluor 568
B-1000	Vector Laboratories, Burlingame, CA	Rabbit IgG	Biotin

Sciatic and optic nerves were dissected from two adult males following brain removal and fixed in 4% paraformaldehyde for 20 minutes, then transferred to PBS. Optic nerves were cryoprotected in a 20% sucrose solution overnight, then cryosectioned at 10 μm thickness and mounted on microscope slides. Sciatic nerves were mechanically desheathed in PBS, teased, and dried on microscope slides.

Nerve tissue

Prior to immunostaining, sciatic nerve slides were frozen on dry ice for 5 minutes, then allowed to come back to room temperature. Slides containing nerve samples were postfixed and permeabilized in methanol at −20°C for 20 minutes. A liquid repellent border (Liquid Blocker; Ted Pella, Redding, CA) was drawn along the edges of the slide, and then the samples were rehydrated with phosphate buffer (pH 7.4; PB). Samples were blocked with 10% goat serum diluted in PB with 0.1% Triton-X and 1% glycine for 1 hour, then incubated with the anti-Cntnap2 antibody diluted to 1:500 in blocking solution overnight at 4°C. After washing with PB, samples were incubated with antirabbit Alexa Fluor 488 (Table 2) at 1:1,000 in blocking solution for 4 hours. The procedure was then repeated with anti-Kvβ2 (Table 1) at 1:250 and antimouse Alexa Fluor 568 (Table 2) at 1:1,000. Glass coverslips were mounted on slides using ProLong Gold antifade reagent (Life Technologies).

Brain sections

One of the replicate sets of brain sections from each bird was used to identify those that contained song control nuclei, using 1% thionin staining to reveal cytoarchitecture. In some cases, sections were alternatively incubated with NeuroTrace fluorescent Nissl stain (Life Technologies) diluted at 1:200 in 0.1 M PB for 20 minutes. For quantification of Cntnap2-positive neurons in RA, slides were chosen with those sections that contained the largest cross-sectional area of RA, in order to control for position within the nucleus, and thawed to room temperature. A liquid repellent border was drawn along the edges of the slide, and then the sections were rehydrated with PB. Endogenous peroxidases were quenched with 0.05% hydrogen peroxide diluted in PB for 30 minutes. Sections were incubated with 5% goat serum in PB containing 0.1% Triton-X for 1 hour. Anti-Cntnap2 antibody was diluted to 1:1,000 in PB and applied to the sections overnight at 4°C. Sections were then incubated at room temperature with a biotinylated goat antirabbit secondary antibody (Table 2) at 1:200 in PB for 1 hour, washed, then incubated with avidin-biotin complex (VECTASTAIN Elite ABC Kit (Standard*), Vector Laboratories, Burlingame, CA) at 1:200 in PB with 0.1% Triton-X for 90 minutes. Sections were stained with fluorescein- or rhodamine-tyramide (Hopman et al., 1998) at 1:1,000 in PB with 0.1% Triton-X and 0.003% hydrogen peroxide. For double labeling, following Cntnap2 immunostaining, sections were incubated overnight at 4°C with either anti-NeuN or anti-parvalbumin antibodies (Table 1) at 1:1,000 in PB. Sections were then incubated at room temperature for 4 hours with antimouse Alexa Fluor 488, 555, or 568 (Table 2) diluted at 1:1,000 in PB. In the hippocampus, tyramide signal amplification was used for both labels. As above, peroxidase activity was quenched and sections were incubated with anti-NeuN at 1:500, then with antimouse HRP at 1:1,000 for 2 hours. These sections were then stained with rhodamine-tyramide as previously described. Peroxidases were quenched again with 0.3% hydrogen peroxide and Cntnap2 immunostaining followed as described above. Slides were mounted with glass coverslips using ProLong Gold antifade reagent (Life Technologies).

Surgical procedures

General methods

Adult male zebra finches were anesthetized with 2–4% isoflurane carried by oxygen using a Universal Vaporizer (Summit Anesthesia Support, Menlo Park, CA) for the duration of the surgery. The bird was placed on a homeothermic blanket mounted in a stereotaxic apparatus at a 45° head angle from the center of the ear bars to the tip of the beak. The cranial feathers were removed to expose the scalp, which was then cleansed using povidone-iodine. In order to preserve vascular

flow to the region, a semicircular incision was made originating and terminating at the caudal edge of the exposed scalp. The scalp was then folded back over a Gelitaspon (Gelita Medical, Amsterdam, Netherlands) moistened with sterile saline to expose the skull. Injections and recordings, described below, were made through ~1 mm^2 windows cut in the skull. After each procedure the scalp was closed and sealed with Vetbond (Fisher Scientific).

Retrograde targeting of RA projection neurons

An ~1 mm^2 window was cut into the skull over the cerebellum ~0.4 mm from the midline, bilaterally. A carbon fiber electrode (Kation Scientific, Minneapolis, MN) was lowered into the brain 4.0 mm below the surface. Multiunit activity was amplified (A-M Systems, Sequim, WA), filtered (300 Hz highpass, 5 kHz lowpass), digitized at 20 kHz (Micro1401, CED, Cambridge, UK), and recorded with Spike 2 software (CED). The location of nXIIts was determined by moving the electrode until multiunit activity corresponded to respiratory expiration. The carbon fiber electrode was then replaced with a glass electrode filled with Green Retrobeads IX (Lumafluor, Naples, FL). Retrobeads were injected into nXIIts with a picospritzer (Toohey, Fairfield, NJ) 3 times on each side for 30 ms at 20 psi. Six days after the procedure each bird was euthanized and perfused with paraformaldehyde as described above.

LMAN lesions

A window was cut in the skull 5.15 mm rostral and 1.7 mm lateral of the midsagittal bifurcation for a unilateral injection. A glass electrode was filled with 10 mg/mL ibotenic acid (Fisher Scientific) in PB, pH 7.0, and lowered into the brain 2.0 mm from the surface to target LMAN and 96.6 nL were injected using a Nanoject II (Drummond Scientific, Broomall, PA). Four days after injection, birds were euthanized and brains collected and sectioned as described above. Sections containing LMAN were stained with thionin as described above to verify the extent of the lesion.

Cntnap2 protein quantification and analysis

Images were acquired using an Axio Imager.A1, with an AxioCam HRm digital camera or LSM 410 laser scanning confocal imager attached to an Axiovert 100 (Carl Zeiss, Oberkochen, Germany). Axiovision software (Carl Zeiss) was used to optimize photomicrographs to remove background, improve brightness and contrast, and to pseudocolor the images. For consistency, Cntnap2 is always represented here in green despite the true color of the fluorophore. In most cases, adjustments were made to the entire image and not to selective subregions, with the exception of the photomicrographs in Figure 2, in which artifacts of the immunostaining were removed. Anatomical regions were identified according to the published stereotaxic zebra finch brain atlas (http://www.ncbi.nlm.nih.gov/books/NBK2348/, courtesy of Dr. Barbara Nixdorf-Bergweiler and Hans-Joachim Bischof). ImageJ (Rasband, 1997–2012) was used to quantify Cntnap2-positive cells as follows. First, a border was drawn around RA based on the density of NeuN immunoreactivity. For areas outside of RA, a 600-pixel diameter circle was drawn laterally from RA in either the dorsal (AD) or ventral (AIV) part of the arcopallium. Within the border, all NeuN and Cntnap2-positive cells were counted. The total counts for each signal were adjusted using the Abercrombie method (Guillery, 2002) to reduce errors due to the 2D counting method. Each count was multiplied by the tissue thickness (T) and divided by the thickness plus the average diameter of the objects counted (T+d). This adjustment (T/(T+d)) was calculated separately for each section analyzed, and reduced the raw counts by 11–33%, with an average of 24%. To control for the different sizes of RA across sections and animals, statistical significance was determined by nonparametric resampling (bootstrapping) of the ratios of Cntnap2 to NeuN counts. This was done in two stages. First, a modified two-way analysis of variance (ANOVA) compared sex, age, and the interaction effect. A Fisher's F statistic was calculated for each of the groups, then the groups were pooled and data were sampled with replacement 10,000 times, generating a range of pseudo-F statistics. Statistical significance was achieved when the F statistic from the real data was greater than 95% ($P < 0.05$) or 99% ($P < 0.01$) of the pseudo-statistics. Then, for groups with an ANOVA *P*-value below 0.05, modified Student's *t*-tests were performed for individual groups with the same resampling protocol as described for ANOVA, instead using a Student's *t* statistic.

RESULTS

Antibody validation

Bioinformatic analysis revealed that the C-terminus of Cntnap2 is highly conserved between humans and zebra finches (Panaitof et al., 2010), and that the last 76 amino acids are identical (amino acids 1255–1331 in human, 1252–1328 in zebra finch: GVNRNSAIIGGVIA VVIFTILCTLVFLIRYMFRHKGTYHTNEAKGAESAESADAAIMN NDPNFTETIDESKKEWLI). A commercial antibody available from Millipore and raised against C-terminus amino acids 1315–1331 of human CNTNAP2 (1312–1328 of zebra finch Cntnap2) was thus selected to test its

ability to specifically detect the zebra finch isoform. In western analyses of zebra finch whole brain homogenate, this antibody detects a single prominent band at the predicted molecular weight of ~180 kDa. Preadsorption of the antibody with the antigenic peptide considerably decreases the intensity of this band (Fig. 2A). The specificity of the antibody was further validated by overexpressing zebra finch Cntnap2 (accession number NM_001193337.1) in ZFTMA cells (Itoh and Arnold, 2011), a zebra finch immortalized cell line that does not endogenously express the protein. Cultures transfected with zebra finch Cntnap2 produce the same protein band, whereas those from untransfected cultures (not shown) or transfected with a control construct containing sequences coding only for GFP do not (Fig. 2B). Specificity of the antibody was again confirmed by preadsorption (see Materials and Methods).

The Millipore antibody was subsequently tested for use in immunohistochemistry. In mammals, Cntnap2 is expressed in axons of myelinated nerves, colocalized with VGKC subunits (Poliak et al., 1999, 2001, 2003; Gu and Gu, 2011). To verify that the Millipore antibody detects zebra finch Cntnap2 in situ, we immunostained optic (Fig. 2C–E) and sciatic (Fig. 2D–F) nerves dissected from zebra finches for both Cntnap2 and Kvβ2. In both nerve preparations, the signals from the two antibodies overlap, as evidenced by the colocalization tools in ImageJ, further confirming that the antibody specifically detects zebra finch Cntnap2.

Cntnap2 protein distribution in the zebra finch brain

Similar to reported mammalian data (Poliak et al., 1999), Cntnap2 distribution is extensive in zebra finch brains, although not expressed to the same level in all regions. Particular enrichment is observed in myelinated areas consistent with axonal expression, such as the fronto-arcopallial tract, optic tract, optic chiasm (not shown), the lateral forebrain bundle (Fig. 3A–C), and layer 5 of the optic tectum (Fig. 3D–F). In the cerebellum, the Purkinje cell layer is marked by intense Cntnap2 immunostaining of cell bodies, and fibers containing Cntnap2 can be observed in the cerebellar white matter. Much less Cntnap2 is found in the granular and molecular layers (Fig. 3G–I). In the midbrain, Cntnap2 is found in the parvocellular portion of the isthmus nucleus (not shown). Thalamic regions containing high levels of Cntnap2 include the anterior dorsomedial nucleus, dorsal portion of the lateral mesencephalic nucleus, rotund nucleus, lateral spiriform nucleus, and pretectal nucleus. In the telencephalon, enrichment of Cntnap2 is found in the entopallium, the anterior hyperpallium, striatopallidum, globus pallidus, field L (not shown), and cell bodies resembling pyramidal neurons (Montagnese et al., 1996) in the medial hippocampus (Fig. 3J–L).

Within the song circuit of an adult male zebra finch, Cntnap2 protein distribution generally follows the mRNA distribution reported in Panaitof et al. (2010), with some exceptions. Although cortical nucleus HVC does contain Cntnap2-positive cells, expression is not enriched relative to the surrounding nidopallium (Fig. 4A–C). As with the mRNA, cortical nuclei RA (Fig. 4D–F) and LMAN (Fig 4G–I) have elevated Cntnap2 levels relative to the surrounding arco- and nidopallium, respectively. In contrast with the reported mRNA levels, the basal ganglia song control region, area X, exhibits greater Cntnap2 protein expression than the surrounding striatopallidum (Fig. 4J–L). The Cntnap2 protein in the aforementioned areas is present not only on cell bodies, but also in the neuropil. The thalamic song nucleus DLM, however, has Cntnap2-positive cell bodies, but relatively less protein in the neuropil than the surrounding thalamic regions (Fig. 4M–O).

Sexually dimorphic expression of Cntnap2 in RA

Cntnap2 mRNA expression is sexually dimorphic in LMAN and RA in developing zebra finches. Males have slightly more *Cntnap2* in LMAN than females throughout development, although the level of expression increases in both sexes with age. There is a more striking difference in expression in RA at 50d. Similar *Cntnap2* levels are detected in both sexes prior to 35d. Between the two timepoints, expression in females begins to decrease, while males maintain a high level through adulthood (Panaitof et al., 2010). We therefore compared levels of Cntnap2 immunostaining in RA in both sexes at developmental timepoints within sensory acquisition and sensorimotor learning periods, and after song crystallization (males, Fig. 5A–E; females, Fig. 5F–J). At 25 and 35d leading up to the onset of sensorimotor learning, the fraction of RA neurons that are positive for Cntnap2 are comparably enriched in both sexes relative to the surrounding dorsal and ventral intermediate arcopallium (AD and AIV, respectively). However, by 50d the fraction of Cntnap2-positive neurons in female RA significantly decreases (Fig. 5L), and falls to levels comparable to those in AD and AIV (Fig. 5M,N). This timepoint falls within the sensorimotor phase of vocal learning, during which males practice their memorized song (Eales, 1985). Male Cntnap2 enrichment in RA is maintained throughout development and into adulthood and crystallization of song, whereas in females it is

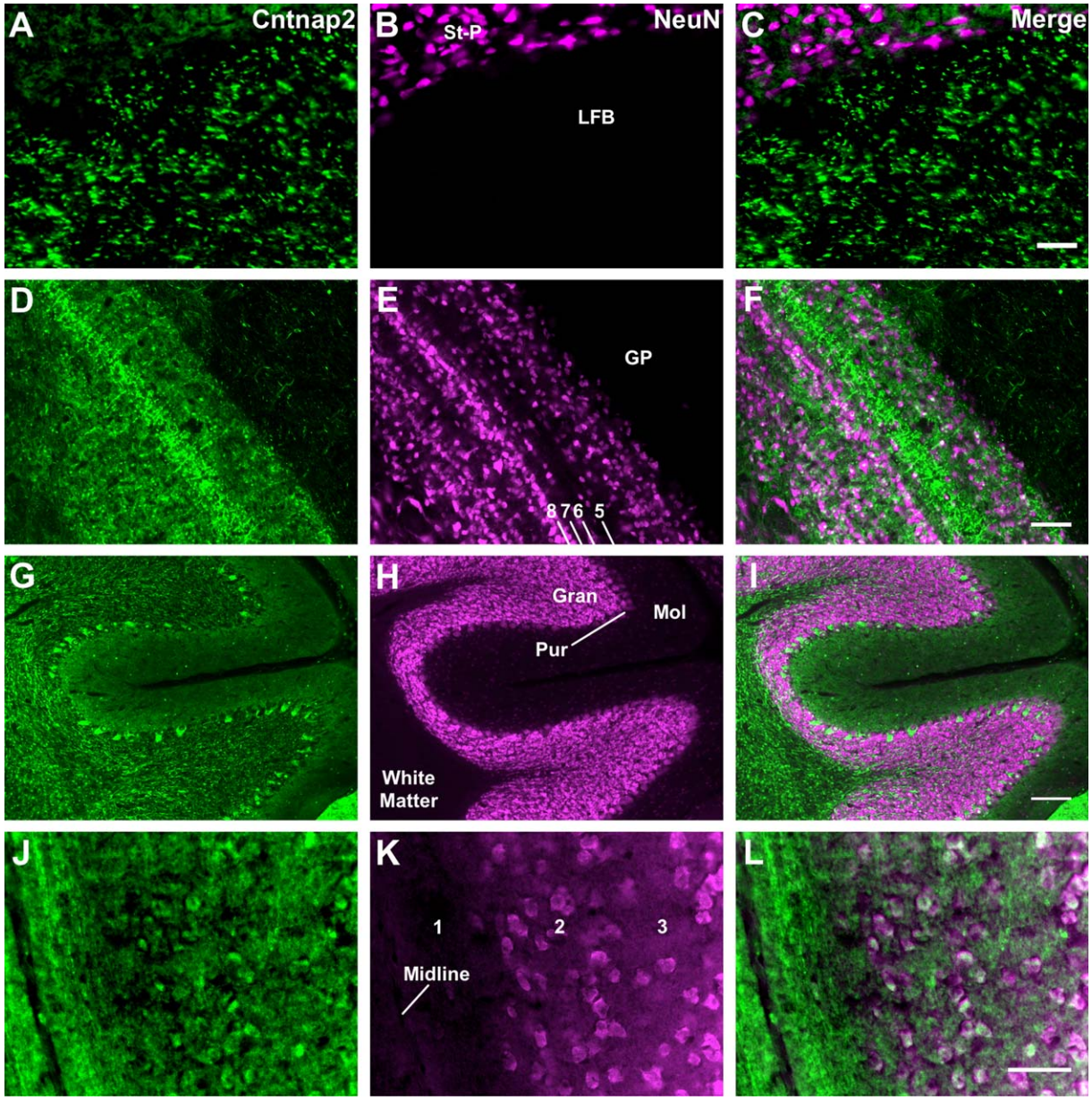

Figure 3. Cntnap2 distribution in nonsong circuit brain regions. Cntnap2 is detected in several areas outside the song circuit of the zebra finch brain, including in structures reported to express Cntnap2 in rodents (Poliak et al., 1999). Nonsong circuit tissue in this figure are taken from regions depicted in Figure 1B. Neuron-specific marker NeuN (magenta) is used for reference. **A–C:** Axonal patterning of Cntnap2 label in the lateral forebrain bundle within the telencephalon. **D–F:** intense Cntnap2 (green) labeling along axons in layer 5 of the optic tectum. Numbers in (B) indicate the layers of the optic tectum according to Ramón y Cajal (1911). **G–I:** Purkinje cell bodies and the cerebellar white matter strongly express Cntnap2, with less in the molecular layer, and fibrous signal in the granular layer and white matter. **J–L:** Coronal section of the medial hippocampus; numbers indicate layers (Montagnese et al., 1996). Cntnap2 marks neuronal somata in the pyramidal cell region (white arrows). See list for abbreviations. Scale bars = 50 μm in A–C; 200 μm in D–L. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significantly reduced. The difference in Cntnap2 protein expression within the arcopallium between males and females and at different developmental stages appears unique to RA. A comparison of the number of Cntnap2-enriched cells in AD and AIV reveals no significant effect of age or sex (Fig. 5M,N).

LMAN projections contribute to Cntnap2 expression in RA

To test the possible contribution of LMAN terminals to Cntnap2 in RA, LMAN was unilaterally lesioned using ibotenic acid in three adult males (Fig. 6A–C, D–F, G–I). The resulting Cntnap2 protein expression was observed

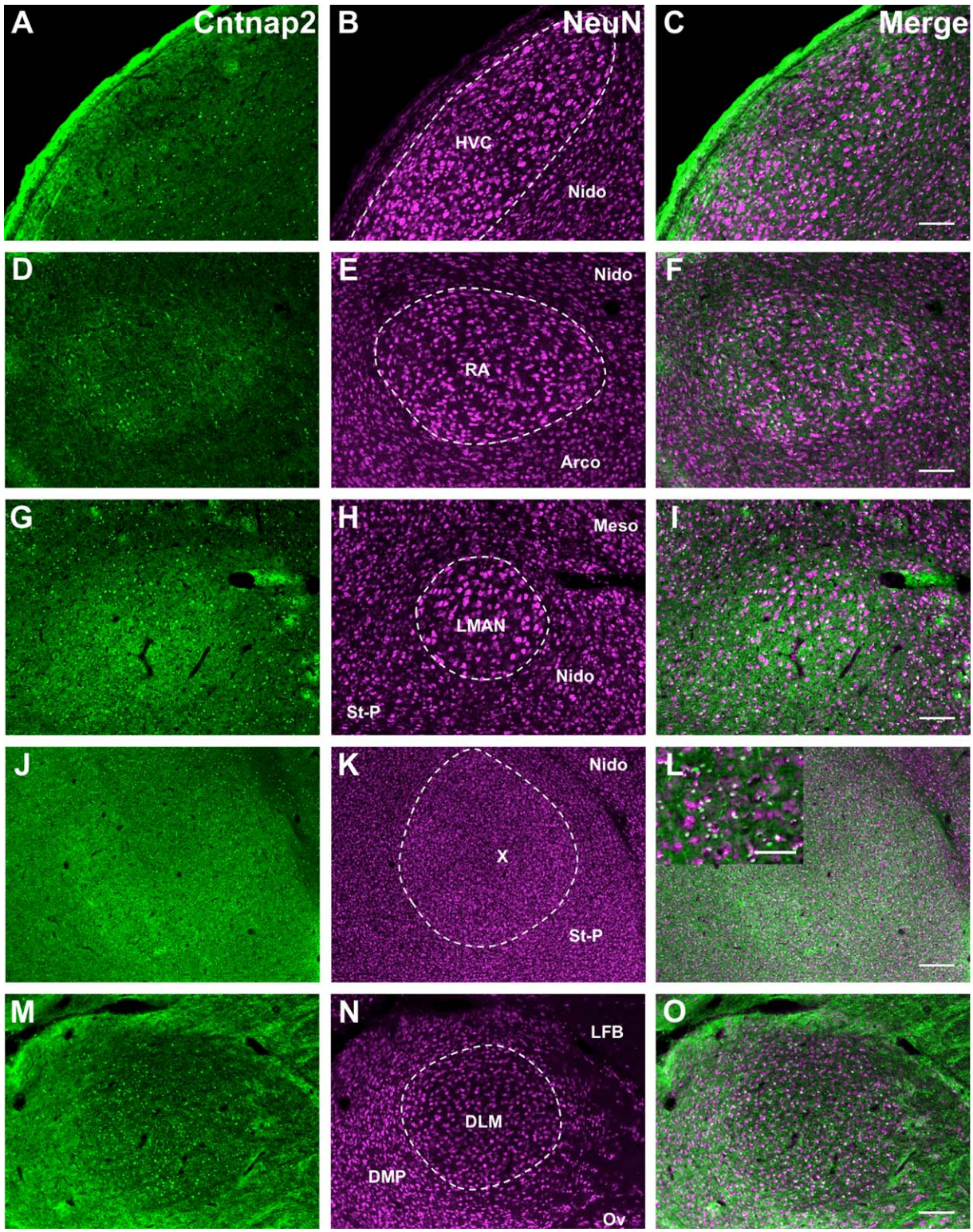

Figure 4. Cntnap2 protein in song circuit nuclei. Fluorescent photomicrographic images of song control nuclei. Cntnap2 signals are in green, and NeuN signals in magenta. **A–C:** HVC in the nidopallium; **D–F:** RA in the arcopallium; **G–I:** LMAN in the nidopallium; **J–L:** Area X in the striatopallidum, inset: higher magnification inside X. **M–O:** DLM in the thalamus, along with the ovoid nucleus, the dorsomedial nucleus of the posterior thalamus, and the lateral forebrain bundle. Each nucleus is indicated by dashed line traces on the NeuN (middle column) panels. Greater Cntnap2 labeling is found within RA, LMAN, and area X relative to surrounding brain regions on both cell bodies and in the neuropil. HVC and DLM contain Cntnap2-expressing cells, but with expression levels comparable to their surrounding tissues. See list for abbreviations. Scale bars = 200 μm A–I; in 100 μm in J–L (50 μm inset); 200 μm in M–O. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

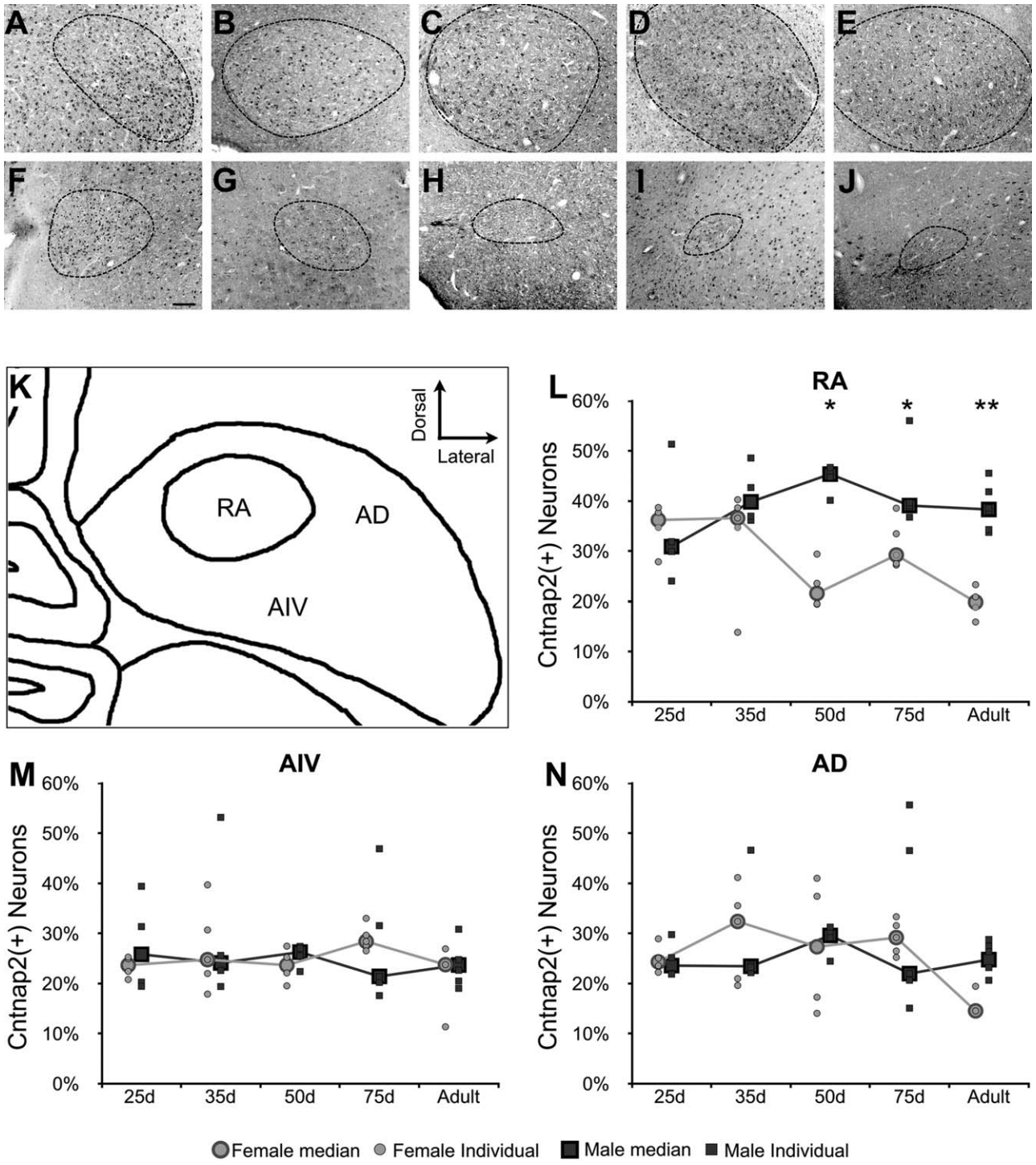

Figure 5. Cntnap2 within RA in both sexes at developmental timepoints during male song learning. **A–J:** Representative images of Cntnap2 immunolabeling of cells in male (A–E) and female (F–J) RA at timepoints during development encompassing the onset of sensory acquisition, sensorimotor learning, and crystallization of song. Anti-NeuN signals (not shown) were used to trace the border of RA in each image. As previously reported (Konishi and Akutagawa, 1985; Nixdorf-Bergweiler, 1996), the size of RA begins to decrease in females and increase in males starting around 35d and continues through development until maturity. **K:** A diagram of RA and the two arcopallial regions in which labeled cells were counted: the ventral intermediate arcopallium (AIV) and the dorsal arcopallium (AD). **L–N:** Graphs representing the percentage of Cntnap2-positive neurons out of the total number of NeuN-positive cells found in RA, AIV, and AD, respectively, for 3–6 birds of each sex at each timepoint. Statistical significance was determined by resampling ANOVA, followed by individual Student's *t*-tests $*P < 0.05$, $**P < 0.01$. See list for abbreviations. Scale bar = 100 μm.

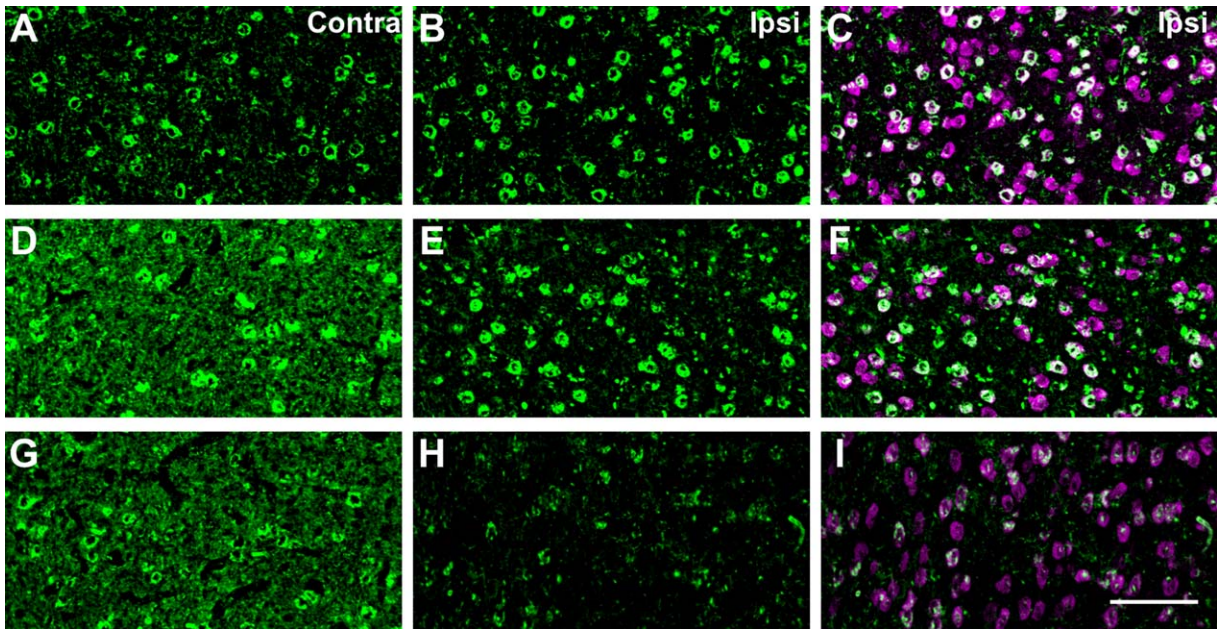

Figure 6. Unilateral LMAN lesions result in an ipsilateral decrease of Cntnap2 in RA. Representative photomicrographic images of Cntnap2 labeling (green) in RA from three adult male zebra finches (**A–C, D–F, G–I**) in which LMAN was lesioned unilaterally by injection of ibotenic acid. Double labeling with NeuN (magenta; C,F,I) indicates neuronal cell bodies. In all cases, the lesion reduces the amount of Cntnap2 in the neuropil, but not cell bodies, in ipsilateral RA relative to the contralateral nucleus, indicating that some of the Cntnap2 in the neuropil originates from LMAN projections. See list for abbreviations. Scale bar = 25 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in the ipsilateral RA and compared to that in the nonlesioned contralateral side. Somatic expression of Cntnap2 remained unaffected in the ipsilateral RA, but there was a decrement in immunostaining intensity in the neuropil compared with the contralateral RA, suggesting that some of the Cntnap2 is indeed from LMAN projections. In summary, within the vocal production circuit, Cntnap2 enrichment appears to be most prominent in RA and due to expression in both neuronal somata and neuropil, including that arising from within LMAN nerve terminals.

Cntnap2 is expressed in RA projection neurons

Within RA, Cntnap2 somal expression is restricted to a subset of the neuronal phenotypes. At least two distinct neuronal populations in RA have been defined based on their electrophysiological signatures and morphology: GABAergic interneurons, and glutamatergic projection neurons (Spiro et al., 1999). The latter directly synapse onto neurons within nXIIts, which directly innervates the syrinx. Parvalbumin staining has been used to differentiate these two types. Whereas some interneurons stain intensely for parvalbumin, projection neurons stain relatively weakly (Wild et al., 2001). To determine whether Cntnap2 is expressed in projection neurons, fluorescent retrobeads were injected into nXIIts (Fig. 7A–C). Following retrograde transport, fluorescent signals colocalized with Cntnap2 signals in RA (Fig. 7D–F), but not in cells that expressed a high level of parvalbumin (Fig. 7G–I). Rather, we found that Cntnap2 signals overlapped only with weakly parvalbumin-positive neurons, consistent with the interpretation that RA projection neurons express Cntnap2 (Fig. 7J–L). The overlap of retrobeads with Cntnap2 signals further supports the hypothesis that Cntnap2 is expressed in RA neurons which project to nXIIts.

DISCUSSION

Here we have characterized the protein distribution of Cntnap2, a molecule linked to human language disorders, in the brain of a nonhuman vocal learner, the zebra finch species of songbird. Because the neurons that are dedicated to vocal learning are clustered together in the songbird brain (Fig. 1), this analysis enables direct comparison of Cntnap2 levels within song-dedicated neurons relative to their levels in surrounding tissues, which, although made up of similar cell types, contribute to nonvocal-related functions. Moreover, the sexual dimorphism of vocal learning and the underlying song control circuitry allow us to compare protein expression between vocal and nonvocal learners within the same species.

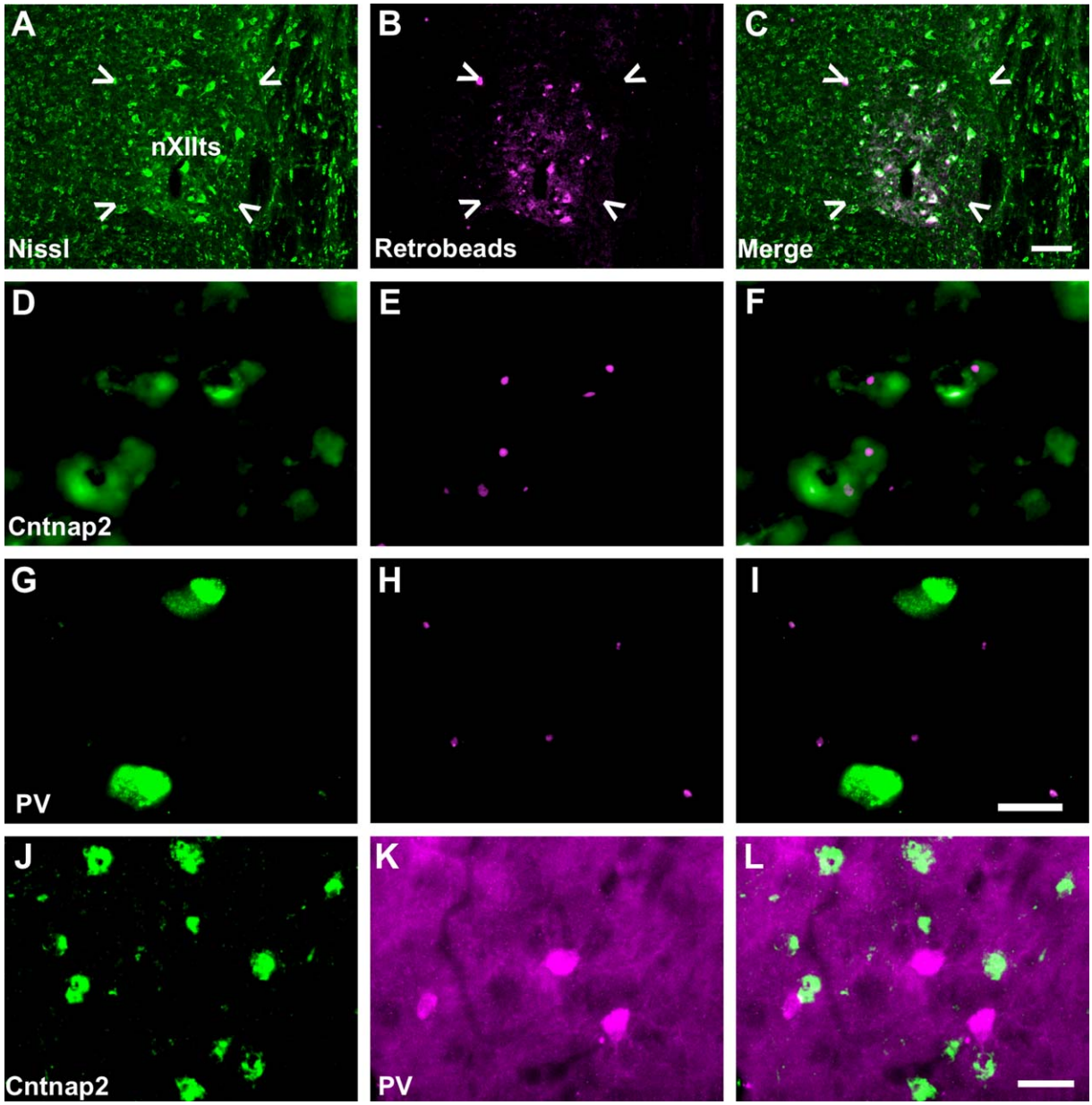

Figure 7. Cntnap2 is expressed in RA projection neurons, not parvalbumin-positive interneurons. **A–C:** Injection site of retrobeads (magenta) in nXIIts (indicated by white arrows), identified by Nissl stain (green). **D–F:** Retrobeads overlap with Cntnap2 (green) expressing cells in RA. **G–I:** Retrobeads do not overlap with strongly parvalbumin positive interneurons. **J–L:** Cntnap2 immunolabeling (green) does not overlap with RA inhibitory interneurons intensely labeled with parvalbumin (magenta). Retrograde labeling reveals that RA projection neurons express Cntnap2 and confirms its absence in parvalbumin-positive interneurons. See list for abbreviations. Scale bars = 50 μm in A–C; 20 μm in D–I; 25 μm in J–L. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

We can further draw parallels between humans and songbirds by investigating the cell types within a song nucleus in which we detect Cntnap2 expression.

Outside the song circuit, immunoreactivity is widespread throughout the telencephalon with areas of particularly high expression, such as in myelinated regions, and in the Purkinje cell layer of the cerebellum, and in pyramidal-like cells (Montagnese et al., 1996) in layers 2 and 3 of the hippocampus (Fig. 3), similar to that described for mammals (Poliak et al., 1999). Notably, however, expression within several nuclei of the song circuit in the adult brain is strikingly different than in their respective surrounding regions, which are not part of the song control circuitry (Fig. 4). In the AFP, Cntnap2 protein is enriched in cortical LMAN relative to the anterior nidopallium, in area X relative to the striatopallidum, and in the somata of DLM relative to the anterior thalamus. Although enrichment in LMAN and

DLM is expected based on the mRNA data, the enrichment in area X is surprising given the lower transcript levels in this region relative to the surrounding striatopallidum (Panaitof et al., 2010). Cntnap2 protein is found in the neuropil of area X, leaving open the possibility that some of the protein arises from HVC and/or LMAN terminals, similar to the contribution of LMAN to Cntnap2 expression in RA (Fig. 6). There is also somal Cntnap2 expression, suggesting at least some protein originates in area X. The difference between the observed mRNA and protein data may reflect state-dependent regulation of the protein, perhaps by transcription factors such as FoxP2 (Teramitsu and White, 2006; Miller et al., 2008). Whether Cntnap2 is a direct target of FoxP2 in zebra finches, as it is in humans (Vernes et al., 2008), remains to be tested. The zebra finch genomic Cntnap2 sequence (RefSeq assembly ID GCF_000151805.1) contains many potential FoxP2 binding sites (Stroud et al., 2006), mostly in the first intron. The FOXP2 binding site in humans was confirmed to be in the first intron by chromatin immunoprecipitation (Vernes et al., 2008). The lower mRNA levels and higher protein in area X thus likely reflect a combination of cellular trafficking, transcriptional and posttranscriptional regulation. Whatever the mechanism, Cntnap2 mRNA and protein expression within the nucleus differs from levels in the surrounding tissue, despite the similar cell type composition of these subregions.

Cntnap2 protein distribution in the posterior pathway is similar to that for the mRNA. The amount within HVC is comparable to the surrounding nidopallium, whereas it is enriched in RA of males and juvenile females (Panaitof et al., 2010). The connectivity of the posterior vocal pathway in males suggests that RA-projecting neurons in HVC are analogous to mammalian neurons in cortical layer 2/3, which do not show prominent Cntnap2 staining, whereas RA projection neurons are analogous to mammalian cortical layer 5 pyramidal neurons (Jarvis, 2004), which exhibit prominent Cntnap2 levels (Poliak et al., 1999). The projection from RA to nXIIts is a corticospinal connection shared with mammalian motor cortex and is hypothesized to allow direct activation of individual muscles necessary for fine motor control (Vicario, 1991). Notably, these direct connections onto motor neurons controlling the muscles involved in phonation are posited to enable the vocal learning capacity of select species such as humans and songbirds (Jürgens, 2009; Arriaga et al., 2012). Overlap of retrobeads injected into nXIIts in RA and Cntnap2-positive neurons (Fig. 7) indicates that Cntnap2 is present in this connection, raising the possibility that Cntnap2 is required for its establishment and/or proper function. Additionally, the reduction of Cntnap2 in the neuropil of RA following an ipsilateral LMAN lesion (Fig. 6) suggests that some of the enrichment in RA is provided from LMAN projections. This long-range connection is reminiscent of the connectivity that is altered in humans bearing the *CNTNAP2* risk alleles for ASD and SLI who exhibit increased local and decreased long-range connectivity of the medial prefrontal cortex (mPFC), and less lateralization than their nonrisk allele counterparts (Scott-Van Zeeland et al., 2010). In fact, LMAN is postulated to be homologous to human PFC based on shared physiologic and anatomic features including connectivity (Kojima et al., 2013). Taken together, these parallel observations in humans and songbirds support the idea that Cntnap2 affects neural connectivity critical for vocal learning across taxonomic classes.

This hypothesis is further supported by the sexually dimorphic expression in zebra finch brain. Similar to that reported for *Cntnap2* mRNA, males and females share protein enrichment in RA early in development. However, by 50d the enrichment in female RA wanes, whereas it persists in males throughout adulthood. Since Cntnap2 is similarly enriched in RA in males and females prior to 50 days, the sexual dimorphism may reflect a change in cell composition in RA or a sex-based difference in gene expression within each cell. These data demonstrate a loss of Cntnap2-labeled cells in female RA with age. This may be due to preferential apoptosis (Konishi and Akutagawa, 1990) of neurons that express Cntnap2 or downregulation of both Cntnap2 mRNA and protein in female zebra finches, who do not use this nucleus for producing learned vocalizations. In mammals, some sex-typical behaviors have been associated with sexually dimorphic expression of individual genes, supporting the hypothesis that sex-related behaviors driven by hormones are mediated in part by genes (Xu et al., 2012) or in fact by genes independent of hormones (Arnold et al., 2013). In the case of the zebra finch, genes that exhibit sexually dimorphic expression in song circuitry are likely to be involved, perhaps even crucial, for singing. These same genes may also be involved in human speech and language. This hypothesis was the basis for the prediction that FOXP1 mutations would impair human speech. FOXP1 is a transcription factor closely related to FOXP2, and the two form heterodimers to control gene expression. Sexually dimorphic expression of *FoxP1*, but not *FoxP2*, was found in the song circuit of quiescent zebra finches, leading to the aforementioned prediction (Teramitsu et al., 2004). Subsequently, several cases were described of FOXP1 mutations in people with language disorders (Pariani et al., 2009; Carr et al., 2010; Hamdan et al., 2010; Palumbo et al., 2012). The sexually dimorphic expression of Cntnap2 in RA also fits this pattern, and may in fact be regulated by FoxP1 in tandem or independent from FoxP2.

What might be the mechanistic function of Cntnap2 in the song circuit, or RA specifically? Cntnap2 is closely related to the neurexins, which have also been implicated in ASD (Südhof, 2008). Although neurexins function at the synapse, Cntnap2 is found at the juxtaparanodes of myelinated axons. There, it is responsible for the clustering of *Shaker*-type VGKCs (Poliak et al., 1999, 2003; Horresh et al., 2008). Selective blockade of these channels on axons from rat central nervous system during myelination early in development leads to aberrant action potential waveforms. However, when the animal becomes mature application of the blocker no longer affects the waveform (Devaux et al., 2002). In songbirds, all song circuit nuclei send and receive long-range connections, which may require Cntnap2 at a macrocircuit level to cluster VGKCs at juxtaparanodes in order to establish and/or maintain synaptic connections required for learning and producing vocalizations. Loss of Cntnap2 in the neuropil of RA following LMAN lesion is evidence for a macrocircuit role for Cntnap2 in this cortical–cortical connection. This suggests that if the role of Cntnap2 in clustering VGKCs is important for vocal learning, it will have the greatest impact early in development, while the process of myelination is still ongoing. Cntnap2 may have additional, yet unknown functions, suggested by *CNTNAP2* enrichment in human embryonic cortex well before myelination (Abrahams et al., 2007). Recent evidence suggests that Cntnap2 may influence synaptic connectivity, increasing cell-autonomous dendritic arborization and the number of synaptic sites in cultured neurons. Contactin 2, the binding partner of Cntnap2, appears to have the opposite effect on synaptic connectivity (Anderson et al., 2012). Contactin 2 and Cntnap2 together may affect the development of brain areas related to vocal learning and language. Cntnap2 may be important for microcircuit connectivity in song nuclei of the adult zebra finch brain as well, by establishing and maintaining local connections within each nucleus through increasing dendritic arborization and the number of active postsynaptic connections. According to this hypothesis, we expect that loss of Cntnap2 in male RA before the onset of sensorimotor learning would lead to fewer connections with HVC and an impaired ability to mimic the tutor's song.

Further investigation into the role of Cntnap2 in vocal learning in songbirds will certainly benefit our understanding of human speech disorders associated with risk variants of the gene, as well as the neurobiology of language as a whole. Taking advantage of the well-characterized song circuitry, an individual song nucleus could be targeted for Cntnap2 RNA interference. If Cntnap2 is involved in song learning, as it seems to be in human speech, we expect knockdown to impair vocal learning in juvenile males, whose songs have not yet crystallized. This system may also be used to parse the activational versus organizational effects of Cntnap2 in vocal learning by manipulating Cntnap2 levels at different times during development. Besides behavior, we additionally expect to find neurophysiological changes. Knocking down Cntnap2 in RA may result in a mislocalization of potassium channels, which could slow the repolarization phase of an action potential similar to the effects of blocking those channels, particularly prior to the completion of myelination (Devaux et al., 2002). There may also be changes to synaptic connectivity between RA and HVC or LMAN concurrent with decreased dendritic arborization of projection neurons originating in RA, similar to the effects reported in vitro reported by Anderson et al. (2012). Reducing Cntnap2 levels in LMAN may augment its local connectivity and decrease its long-range connectivity to RA, similar to the altered connectivity in forebrains of humans with risk variants of Cntnap2 (Scott-Van Zeeland et al., 2010). The balance between inhibition and excitation is also likely to be affected, as it is in cases of autism (Cline, 2005) and *Cntnap2* knockout mouse models (Peñagarikano et al., 2011). The present and future investigation into the role of Cntnap2 in vocal learning using songbirds complements studies in mammals moving toward a better understanding of its associated disorders in humans.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

ROLE OF AUTHORS

Both authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study

concept and design: MCC and SAW. Acquisition of data: MCC. Analysis and interpretation of data: MCC. Drafting the article: MCC. Critical revision of the article for important intellectual content: SAW. Statistical analysis: MCC. Obtained funding: MCC and SAW. Administrative, technical, and material support: SAW. Study supervision: SAW.

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COMPARATIVE COGNITION & BEHAVIOR REVIEWS

Recent Advances in the Genetics of Vocal Learning

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Language is a complex communicative behavior unique to humans, and its genetic basis is poorly understood. Genes associated with human speech and language disorders provide some insights, originating with the FOXP2 transcription factor, a mutation in which is the source of an inherited form of developmental verbal dyspraxia. Subsequently, targets of FOXP2 regulation have been associated with speech and language disorders, along with other genes. Here, we review these recent findings that implicate genetic factors in human speech. Due to the exclusivity of language to humans, no single animal model is sufficient to study the complete behavioral effects of these genes. Fortunately, some animals possess subcomponents of language. One such subcomponent is vocal learning, which though rare in the animal kingdom, is shared with songbirds. We therefore discuss how songbird studies have contributed to the current understanding of genetic factors that impact human speech, and support the continued use of this animal model for such studies in the future.

Keywords:
Autism, Basal ganglia, Cntnap2, FoxP1, FoxP2, KE family, Speech, Vocal learning, Zebra finch

Introduction

Vocal learning, which includes the ability to imitate sounds with one's voice, is a rare trait in the animal kingdom. To date, only a few groups of mammals have demonstrated a capacity for vocal learning. These include certain species of echolocating bats, cetaceans, pinnipeds, elephants, and of course, humans (Fitch, 2012; Knornschild, Nagy, Metz, Mayer, & von Helversen, 2010; Stoeger et al., 2012). Outside of mammals, three groups of birds are capable of learning a portion of their vocalizations, namely hummingbirds, parrots, and songbirds, the last of which make up about half of all bird species (Reiner et al., 2004). The disparate pattern of vocal learning across taxa is characteristic of convergent evolution. A parsimonious explanation is thus that preadaptations for vocal learning emerged from non-learning ancestors of each taxon (Fitch, 2011). These preadaptations are likely genetically encoded, which suggests that despite the distant relationships between vocal learners, there are some common genetic factors. One

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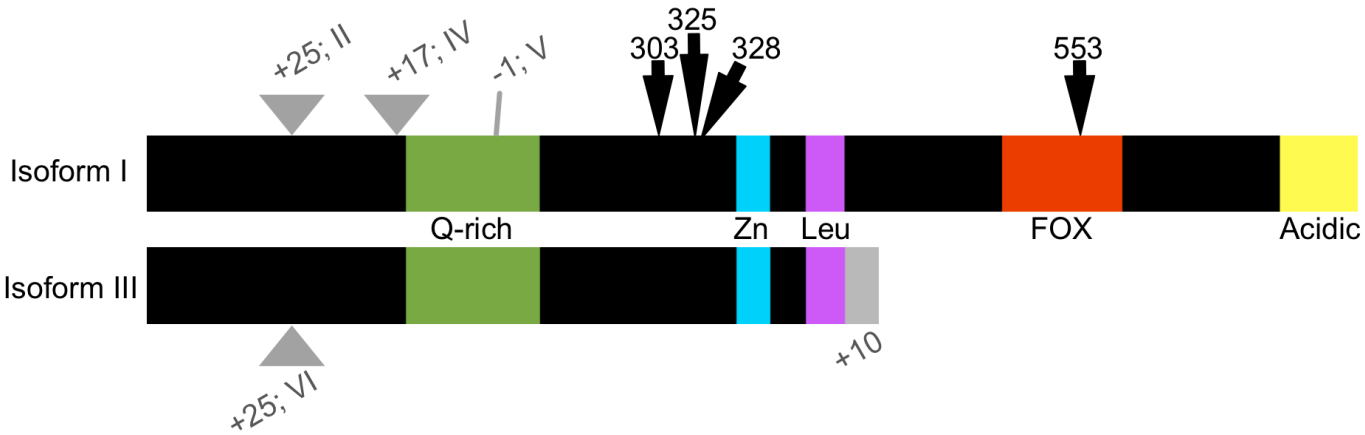
such example is the transcription factor Forkhead Box P2 (FOXP2), which is important for both human and songbird learned vocalizations. This review will discuss the evidence for the involvement of (a) FOXP2 in vocal learning, as well as that for other language-related genes including (b) FOXP1, (c) Contactin Associated Protein-Like 2 (CNTNAP2), (d) Hepatocyte Growth Factor signaling pathway genes, (e) stuttering-related genes, (f) additional genes of interest, and (g) microRNA. For each genetic factor, we will discuss the evidence for its involvement in vocal learning, known mechanisms of action, its role in human speech-related disorders, and animal model studies. Birdsong studies in particular will be reviewed, as the animal behavior that most closely resembles human speech.

1. FOXP2

Human Disease Studies of FOXP2

The transcription factor *FOXP2* was the first gene to be causally linked with language ability. This discovery was made through the study of a human pedigree, referred to as the KE family (Lai, Fisher, Hurst, Vargha-Khadem, & Monaco, 2001), about half of whom suffer from inherited developmental verbal dyspraxia (DVD; also known as childhood apraxia of speech). DVD is characterized by an impaired ability to correctly execute orofacial movements required for speech (Lai et al., 2001; MacDermot et al., 2005). In the KE family, the disorder is inherited in a Mendelian dominant manner, the locus of which was mapped to chromosome 7q31 (Fisher, Vargha-Khadem, Watkins, Monaco, & Pembrey, 1998). An unrelated boy who exhibited the DVD phenotype harbored a genetic disruption in the same region, leading to the identification of *FOXP2* as the cause of the disorder. *FOXP2* codes for a transcription factor found primarily in the brain, lung, and spleen (Shu, Yang, Zhang, Lu, & Morrisey, 2001). The KE mutation results in an amino acid substitution, R553H (Figure 1), in the conserved DNA-binding forkhead box (FOX) region of the protein, which, in in vitro studies, causes abnormal levels of extra-nuclear FOXP2 and impedes its ability to bind to DNA (Mizutani et al., 2007; Vernes et al., 2006). Since the discovery of the relationship between the KE mutation and DVD, other FOXP2 variants have emerged that are associated with speech and language disorders (Feuk et al., 2006; MacDermot et al., 2005; Palka et al., 2012; Raca et al., 2013; G. M. Rice et al., 2012; Shriberg et al., 2006; Zeesman et al., 2006), strengthening the link between FOXP2 and language. (It is important to note here that "language" refers to communication through the use of symbols that are not necessarily verbal, whereas "speech" specifically refers to the spoken component of language.) For example, a non-sense mutation, R328X (Figure 1), was discovered in three related individuals with verbal deficiencies (MacDermot et al., 2005). This mutation results in the loss of the FOX, zinc finger, and leucine zipper domains, the last of which is hypothesized to be crucial for dimerization, which itself aids DNA binding (S. Li, Weidenfeld, & Morrisey, 2004). Despite these strong links to language, *FOXP2* coding variants have not been directly associated with autism spectrum disorder (ASD) or specific language impairment (SLI; Marui et al., 2005; Newbury et al., 2002; Scott-Van Zeeland, Abrahams, et al., 2010; Toma et al.,

Figure 1. Schematic of human FOXP2 isoforms I–VI. FOXP2 is alternatively spliced as two major isoforms: the full-length isoform I and a truncated isoform III. Variations of either major isoform contain inserted or omitted amino acids (II, IV–VI), indicated here as the difference in number of amino acids (gray triangles). Both major isoforms possess a glutamine-rich (Q-rich) area, zinc finger (Zn) and leucine zipper (Leu) domains. Full-length isoforms of FOXP2 also possess a DNA-binding domain and an acid region on the C-terminus. Isoforms III and VI also have an additional 10 amino acids on the C-terminus that are not shared with the full-length isoforms. Arrows indicate amino acid substitutions between human and chimpanzee (303 and 325) or related to human speech disorders (328 and 553).



2013), even though these disorders are also characterized by language deficits. In contrast, within a sample of dyslexic children and their unaffected relatives, a single nucleotide polymorphism (T vs. C) in an intron of *FOXP2*, identified as rs7782412, was correlated with nonword repetition (NWR) score (Peter et al., 2011), with the major allele (T, frequency of 0.558) being associated with impairment on this task. Since dyslexia is associated with impairments of written, but not spoken, language (Lyon, Shaywitz, & Shaywitz, 2003), these data suggest that *FOXP2* aberrations affect language processing as well as spoken motor ability. Notably, language processing deficits and low verbal IQ are symptomatic in the KE family as well (Vargha-Khadem, Watkins, Alcock, Fletcher, & Passingham, 1995), though it is unclear whether these traits are directly related to the *FOXP2* mutation, or are sequelae of DVD.

FOXP2 Function in the Developing Brain

In all animals, the FOX family of transcription factors is involved in regulating biological processes that affect embryogenesis and tissue development, as well as processes underlying adult cancer and aging (Benayoun, Caburet, & Veitia, 2011; Carlsson & Mahlapuu, 2002). FoxP1, 2, and 4 are expressed in embryonic neural tissues (Lu, Li, Yang, & Morrisey, 2002; Shu et al., 2001), and may therefore mediate neurogenesis and/or differentiation. Experimental reduction of Foxp2 in the cortex of embryonic mice through either shRNA or overexpression of the dominant negative KE form of FoxP2 repressed the transition from radial precursor to immediate neuronal progenitor, resulting in decreased cortical neurogenesis (Tsui, Vessey, Tomita, Kaplan, & Miller, 2013). Interestingly, overexpression of human FOXP2 increases neurogenesis, whereas overexpression of murine Foxp2 does not. These data indicate that human FOXP2 exerts a greater neurogenic effect, which is perhaps significant for the construction of the brain, including neural circuits involved in language processing. Foxp2 (here indicating the mouse form of the protein by capitalizing only the first letter, whereas the human form contains all capitals, and camel case for all other chordates) (Kaestner, Knochel, & Martinez, 2000) in conjunction with Foxp4, appears to promote neurogenesis by regulation of N-cadherin (Rousso et al., 2012). In embryonic chick and mouse spinal cord, overexpression of either FoxP increases the release of neural progenitors from the neuroepithelium, whereas knockdown of both prevents this release. These effects have yet to be tested in the cortex.

Another mechanism whereby FoxP2 may promote the development of vocal learning circuitry is through neurite development, especially during embryogenesis. A recent gene ontology study using Foxp2-ChIP and expression arrays found that Foxp2 targets related to neurite development are enriched (Vernes et al., 2011). Using ex vivo neuronal cultures, this study found that expression of wild type *Foxp2* accelerates neurite growth, whereas expression of the KE mutant form has the opposite effect. Ectopic expression of Foxp2, achieved by removing the 3'UTR, which includes its regulatory elements, delays neurite outgrowth *in vitro*, though by seven days neurites form properly (Clovis, Enard, Marinaro, Huttner, & De Pietri Tonelli, 2012).

FOXP2 regulates gene activity by binding to DNA either as a homodimer, or by heterodimerizing with FOXP1 or FOXP4. There are six known isoforms of FOXP2 (Figure 1), two of which are truncated and lack FOX domains (Bruce & Margolis, 2002). The truncated forms, referred to as FOXP2.10+ due to their alternate splicing at exon 10 (Figure 1), do not localize to the nucleus, but may still dimerize with other FOXP2 isoforms (Vernes et al., 2006). Therefore, it is hypothesized that FOXP2.10+ forms act as posttranslational regulators of FOXP2 activity. FOXP2 can also interact with C-terminal binding protein (CtBP) to repress transcription (Li et al., 2004). A new association has been identified between FOXP2 and the gene protection of telomeres 1 (POT1; Tanabe, Fujita, & Momoi, 2011). In cell culture, when POT1 is expressed alone or coexpressed with the KE dominant negative mutation (R553H) of FOXP2, it is not localized in the nucleus. Only when POT1 is coexpressed with wild type FOXP2 is nuclear localization observed. Loss of POT1 can elicit a DNA damage response and cause cell arrest (Hockemeyer, Sfeir, Shay, Wright, & de Lange, 2005). FOXP2, in conjunction with POT1, could therefore affect cell cycling during development. The human phenotype exhibited by the KE mutation may be partly mediated by the inability of the mutant FOXP2 to associate with POT1, thereby disrupting cell cycling during the development of neural tissues subsequently necessary for vocal learning (Tanabe et al., 2011).

Molecular Phylogeny of FoxP2

FoxP2 is highly conserved across species, particularly in the zinc finger and DNA-binding FOX domains (Figure 1). Two amino acid differences between humans and chimpanzees (303N and 325S in the human isoform; Figure 1) are unique to humans among living primates (Enard et al., 2002). Interestingly, these substitutions are shared with extinct hominids such as Neanderthals (Green et al., 2010; Krause et al., 2007; Reich et al., 2010), for whom the capability for language is still uncertain (Benítez-Burraco & Longa, 2012). Between the zebra finch and human isoforms, there are only five additional substitutions, including one in the zinc finger domain, which is conserved in primates and rodents, but differs in the zebra finch ortholog (Teramitsu,

Kudo, London, Geschwind, & White, 2004). Importantly, the DNA binding region is conserved between zebra finches and humans, including the arginine residue corresponding to position 553 in humans that is the site of the KE mutation. There is a considerable amount of homology (>80%) in the zinc finger, leucine zipper, and DNA-binding domains between human FOXP2 and the single FoxP ortholog of fruit flies and honeybees, from which it is believed the vertebrate FoxP family expanded (Kiya, Itoh, & Kubo, 2008; Scharff & Petri, 2011). As in vertebrates, invertebrate FoxP is predicted to be involved in procedural learning and communication, consistent with its neural expression and suggesting that it is most distinct from mammalian FoxP3, which is not associated with neural cell types (Scharff & Petri, 2011). FoxP2 is not well-conserved among echolocating bats nor between bats and other mammals, however, which has been postulated to be the result of a selection pressure on FoxP2 in bats for the evolution of echolocation (Li, Wang, Rossiter, Jones, & Zhang, 2007).

Songbird Studies of FoxP2

Humans are the only living animals that communicate with language (Berwick, Friederici, Chomsky, & Bolhuis, 2013), leaving no single animal model that sufficiently encapsulates every component of the behavior. However, facets of language are shared with other species. Vocal learning is one such facet that is shared with select groups of mammals, but as yet common laboratory models (e.g. rats, mice, nonhuman primates) fail to demonstrate this ability (Arriaga, Zhou, & Jarvis, 2012; Fitch, 2000; Mahrt, Perkel, Tong, Rubel, & Portfors, 2013). Rather, songbirds have been the principal animal models for vocal imitation in a laboratory setting (Panaitof, 2012). Vocal learning in both humans and songbirds relies on connections between the cortex, basal ganglia, and thalamus (Doupe & Kuhl, 1999). An advantage of the songbird model is that the neural structures responsible for vocal production and learning, called song production nuclei, are interconnected and anatomically distinct from the larger neurological subdivisions in which they reside, but are comprised of similar cell types. The song production nuclei are therefore assumed to function similarly to the circuits underlying other forms of procedural learning, but are dedicated to vocal learning. This feature of the songbird neuroanatomy has been incredibly useful for studies of vocal learning genes, many of which are discussed in this review. Among songbirds, zebra finches have been widely used due to their ease of breeding in captivity, as well as the sexual dimorphism of vocal learning (only males sing; Immelmann, 1969) and the song production system, which is incomplete in females (Konishi & Akutagawa, 1985; Nottebohm & Arnold, 1976).

FoxP2 mRNA expression is robust in the basal ganglia of humans and zebra finches (Teramitsu et al., 2004). In the zebra finch striatopallidal song nucleus, area X, FoxP2 transcript and protein levels correlate negatively with early morning singing. FoxP2 protein decreases in area X over the course of two hours when a male directs his songs at a female or when he practices them alone (Miller et al., 2008; Thompson et al., 2013); the latter is referred to as undirected singing. The transcript decreases during the course of two hours of undirected, but not directed, singing (Hilliard, Miller, Horvath, & White, 2012; Teramitsu & White, 2006; Teramitsu, Poopatanapong, Torrisi, & White, 2010). Down regulation of the mRNA is most potent in young birds engaged in sensorimotor learning (Teramitsu et al., 2010) when the more the juvenile practices, the lower his area X *FoxP2* levels. This regulation appears largely due to motor activity, rather than auditory input, as levels also decrease in birds that have been deafened. However, there may be an additional auditory component to this phenomenon, as the degree of down regulation is only correlated with the amount of singing (Hilliard, Miller, Fraley, Horvath, & White, 2012) in juveniles that maintained their hearing (Teramitsu et al., 2010). The distinct behavioral regulation of the mRNA and protein suggests that there is post-transcriptional regulation of FoxP2, at least in the case of directed singing. In any case, both phenomena have been replicated at the two hour time point, namely that the protein levels decline with both directed and undirected singing, whereas the mRNA only declines with undirected song practice. Specifically, new findings show that microRNAs that target *FoxP2* are up-regulated during undirected, but not directed, singing and lead to corresponding decreases in *FoxP2* mRNA only for the former (Shi et al., 2013).

In their 2013 study of FoxP2 protein expression, Thompson et al. (2013) identified two categories of FoxP2-labeled neurons: those with large nuclei intensely labeled by the FoxP2 antibody, and those with smaller nuclei and weaker labeling. One possibility is that these subtypes represent different stages of maturation within a single population of medium spiny neurons (MSNs). Intensely labeled neurons may be younger neurons either in the process of migrating or already having migrated to area X, whereas weakly labeled neurons may be mature and integrated into the basal ganglia microcircuitry. The intensely labeled neurons peak in density within area X around 35 days and decline with age. The density of weakly labeled 'mature' neurons does not change with age. However, the density of these neurons in area X is behavioral context dependent. Adult males that sing for two hours in the morning exhibit a reduced density of weakly labeled neurons, a finding that replicates the behaviorally modulated levels of FoxP2

described by Fisher et al. (1998) and Miller et al. (2008).

In the zebra finch, experimentally induced reduction of FoxP2 at a developmental stage prior to the onset of vocal motor learning via injection of lentivirus containing an shRNA construct partially impairs the ability to learn the tutor's song (Haesler et al., 2007). Though shRNA-injected young zebra finches are capable of producing sounds similar to those of their tutors, they consistently fail to accurately imitate the tutor's song, often omitting or repeating individual syllables. Additionally, they are unable to accurately imitate the spectral characteristics and timing of the tutor's song. During this period of song learning, new neurons expressing FoxP2, which are hypothesized to affect behavioral plasticity, migrate into area X (Rochefort, He, Scotto-Lomassese, & Scharff, 2007). Surprisingly, though, knockdown of FoxP2 does not prevent the proliferation of new neurons from the ventricular zone. It does, however, reduce the number of dendritic spines on MSNs, suggesting that FoxP2 affects neuronal plasticity without affecting proliferation and migration of new neurons (Schulz, Haesler, Scharff, & Rochefort, 2010). These data provide support for a functional role of FoxP2 in vocal learning subserved by basal ganglia circuits, in addition to mediating the development of the brain regions involved.

Mouse Models of Foxp2

Several mutant mice strains have been generated to study the effects of *Foxp2* on brain morphology as well as vocal and nonvocal behaviors. In one such model, the two amino acids characteristic to humans (Enard et al., 2002) were changed to conform to the human sequence (Enard et al., 2009). The resulting mice have altered cortico-basal ganglia circuitry in the form of increased dendrite length in Foxp2-expressing bipolar spiny neurons in layer 6 of the primary motor cortex, MSNs in the striatum, and neurons in the parafascicular nucleus of the thalamus. Long-term depression (LTD) is increased in MSNs of the striatum, and dopamine concentrations are reduced in several brain regions, including the striatum (Reimers-Kipping, Hevers, Pääbo, & Enard, 2011). Despite also expressing the humanlike Foxp2 protein, dendrite lengths of amygdalar and cerebellar Purkinje neurons are unchanged. Purkinje cell LTD is also similar to control levels, which suggests that the humanlike Foxp2 impacts mainly basal ganglia microcircuits (Enard et al., 2009; Reimers-Kipping et al., 2011). In terms of behavior, the mutant mice exhibit decreased exploration, spend more time in groups, and as neonates emit ultrasonic vocalizations with reduced pitch and increased frequency modulation compared to control mice. Interestingly, *FOXP2* knockout heterozygotes with a functional wild type allele have the opposite effects on dopamine levels and behavior (Enard et al., 2009).

Several mouse models have been generated to mimic FOXP2 mutations associated with human disorders. These knock-in mice include murine versions of the KE mutation (R552H; Fujita et al., 2008; Groszer et al., 2008), a similar mutation that results in an amino acid substitution at a different site within the DNA binding domain (N549K; Groszer et al., 2008), and a truncation (S321X) that fails to produce a protein, similar to a human mutation associated with speech impairment (Groszer et al., 2008). These loss of function knock-in mutations are lethal in homozygotes, with mice usually dying within the first month of life, though N549K homozygotes can survive for several months. All knock-in mutants have decreased cerebellar volume and Purkinje cell dendritic arbor (Fujita et al., 2008; Groszer et al., 2008), but otherwise no gross anatomical disturbances were observed in the rest of the brain. Homozygous knockout, R552H, and S321X mutant mouse pups make fewer ultrasonic distress calls, though there are mixed reports about the quality of these vocalizations (Fujita et al., 2008; Gaub, Groszer, Fisher, & Ehret, 2010; Groszer et al., 2008; Shu et al., 2005). Recently, Bowers, Perez-Pouchoulen, Edwards, & McCarthy (2013) investigated these calls using wild type rats and found qualitative and quantitative sex differences. Similar to mice, isolation calls are emitted from rat pups separated from their dam and trigger her to retrieve the pup back to the nest. The authors found that male pups call more frequently, at a lower pitch, and more quietly than do female pups. In turn, the dam responds differently to calls made by each sex, preferentially retrieving male before female pups. Male rat pups have more Foxp2 protein than female pups in several brain areas. Experimental reduction of Foxp2 by injection of siRNA into the ventricles during the first two days of life reverses this sex effect in calling behavior. Treated male pups call less frequently and at a higher pitch than control males. Notably, treatment of female pups with siRNA causes their vocalizations to become male-like, with higher frequency of calling, lower pitch, and lower amplitude. The authors posit that the reversal caused by Foxp2 siRNA is the result of a decrease in Foxp2 in males and a rebound-effect increase in females, although no evidence is provided for the latter. Interestingly, the dam retrieves siRNA-injected females before siRNA-treated males, providing evidence that the retrieval response of the dam depends on the vocal behavior rather than other sexually dimorphic characteristics. This study also finds that, in postmortem human brain tissue, there is more FOXP2 in the cortices of 4-year-old girls than age-matched boys, which coincides with gender-based language differences in children at this age. The authors posit that sex differences in brain FoxP2 levels correlate with the

more 'communicative' sex in human and rodent species.

Since separation calls are not learned (Arriaga et al., 2012) and therefore are not analogous to human speech, studies in *Foxp2* mutants examined other classical learned behavioral skills. One such skill was measured by Morris water maze place learning, in which mice were given four consecutive training trials each day for four days, after which the platform was moved and training began again (Santucci, 1995). Heterozygote knockout mice perform as well as wild types (Shu et al., 2005), indicating that this hippocampal-based learning task is not affected by loss of *Foxp2*. However, R552H mutants are impaired on the accelerating rotarod, a procedural learning task in which mice are placed on a rod that rotates around its axis at an increasing rate and the amount of time before the animal falls from the rod is recorded. Performance on the rotarod relies on basal ganglia activity, suggesting that R552H mutant mice have deficits in activity in these brain regions (French et al., 2012; Groszer et al., 2008). R552H heterozygous mutant mice have corresponding neurophysiological abnormalities, including reduced striatal LTD and increased cerebellar paired pulse facilitation (Groszer et al., 2008). *In vivo* electrophysiological recordings of these mice during the accelerating rotarod learning task show that striatal firing rate activity decreases in R552H mutants, whereas it increases in wild type, and temporal coordination is altered (French et al., 2012). Interestingly, these mutant mice can perform other striatal-based learning tasks, such as pressing a lever for a reward, equally well as controls. These data suggest that Foxp2 activity in the basal ganglia is involved in procedural learning tasks in nonvocal learning species, perhaps in a similar manner to vocal learning in humans and songbirds.

2. FOXP1

FoxP1 is the most similar molecule to FoxP2 and, perhaps not surprisingly, is also linked to human speech. As previously mentioned, FoxP1 and FoxP2 may form heterodimers that regulate transcription in areas where their expression overlaps (Li et al., 2004; Shu et al., 2001; B. Wang, Lin, Li, & Tucker, 2003). Initial support for a role of FOXP1 in vocal learning stems from a study of comparative gene expression in two vocal learners: humans and zebra finches. Unlike *FoxP2*, for which differential expression in song nuclei depends on behavior, *FoxP1* signals constitutively 'mark' the song system, with mRNA enrichment in area X (in males), HVC, and RA relative to their surrounding tissues (Teramitsu et al., 2004). In humans, *FOXP1* and *FOXP2* are found in separate cortical layers: the former is found primarily in layers 2/3 with less expression in deeper layers, whereas the latter is primarily in layer 6 (Ferland, Cherry, Preware, Morrisey, & Walsh, 2003; Teramitsu et al., 2004). Both transcripts are expressed in the human striatum, similar to the expression pattern in the basal ganglia nucleus area X of songbirds. The possible co-regulation of transcription by FoxP members in the songbird song production system, and the comparative gene expression in humans suggested that FOXP1 also plays a role in human language (Teramitsu et al., 2004). Subsequently, Pariani, Spencer, Graham, & Rimoin (2009) reported the first human case of *FOXP1* alteration and speech impairment, in which the patient had a large deletion in chromosome 3 including the *FOXP1* gene. Speech delay was one of several deficits, which also included anatomical and neurological abnormalities. Shortly after this report, several similar cases were published in which patients with *FOXP1* deletions presented cognitive deficits, motor control deficits, speech delay, and autism (Carr et al., 2010; Hamdan et al., 2010; Horn, 2012; Horn et al., 2010; O'Roak et al., 2011; Palumbo et al., 2013; Talkowski et al., 2012). In all the reported cases, however, the language impairment described was more consistent with speech delay than DVD. A screen of patients with DVD failed to identify FOXP1 as a risk factor (Vernes, MacDermot, Monaco, & Fisher, 2009). Though many of the phenotypes associated with mutations in FOXP1 and FOXP2 are non-overlapping, language impairment is common to both (Bacon & Rappold, 2012).

3. CNTNAP2

CNTNAP2 in Human Disease

Similar to the discovery of the relationship between FOXP2 and language through the KE family, a rare mutation in the contactin associated protein-like 2 (*CNTNAP2*) gene was discovered in a genetically related population of Old Order Amish children (Strauss et al., 2006). Some members of this group are afflicted with cortical dysplasia-focal epilepsy (CDFE). The disorder is characterized by the onset of seizures at about 2 years of age, mental retardation, hyperactivity, pervasive developmental delay or autism in the majority of cases, and language regression by the age of 3 in all cases. Patients with CDFE are homozygous for a deletion of a single base pair in *CNTNAP2* exon 22, 3709delG. Subsequent to the initial association between CNTNAP2 mutation and CDFE, it was revealed that it is transcriptionally regulated by FOXP2. In chromatin immunoprecipitation (ChIP) assays, fragments of intron 1 of *CNTNAP2* were bound by FOXP2 at the canonical binding sequence CAAATT (Vernes et al., 2008; Vernes et al., 2011). Mutation of these sites to CGGGTT prevented FOXP2 binding. Overexpression of FOXP2 in the human-derived neuroblastoma cell line SY5Y decreased *CNTNAP2* transcription. To further investigate the relationship between

CNTNAP2 and language ability, variants of the gene were screened in a cohort of families with SLI-afflicted members. Nine intronic SNPs between exons 13 and 15 of *CNTNAP2* correlated with NWR scores. The one SNP most correlated, rs17236239, was also associated with expressive language score. Quantitative transmission disequilibrium testing (QTDT) confirmed a relationship between measures of language ability and four of these SNPs, but failed to confirm a relationship for rs7794745 in a new sample of families containing members with SLI (Newbury et al., 2011). None of the SNPs associated with language-related QTDT measures in a sample of families with dyslexia, indicating that there are separate factors that affect language ability.

Other common *CNTNAP2* polymorphisms have been identified that associate with diagnoses of autism (Arking et al., 2008; Bakkaloglu et al., 2008), for which language impairment is a core deficit, and a language-related measure, age at first word (Alarcón et al., 2008). Interestingly, inherited *CNTNAP2* polymorphisms that are associated with disease occur mainly in introns (Alarcón et al., 2008; Arking et al., 2008), suggesting either these SNPs are in linkage disequilibrium with yet unidentified markers in exons, or the SNPs themselves affect transcriptional regulation of the gene. Quantitative transmission disequilibrium testing revealed a association between the SNP rs2710102 and NWR (Peter et al., 2011). Thirteen *de novo* mutations in *CNTNAP2* have been described in ASD patients that result in an amino acid change in the protein, eight of which were predicted to hinder function (Bakkaloglu et al., 2008). The *de novo* mutations, along with the CDFE mutation identified by Strauss et al. (2006), were investigated further to determine whether they did in fact affect protein function. HEK cells and rat hippocampal neurons were transfected with either wild type human CNTNAP2 or the mutant forms (Falivelli et al., 2012). The mutation associated with CDFE, 3709delG, causes a frameshift that results in the loss of the single transmembrane and intracellular domains of the protein (Figure 2, Strauss et al., 2006). This causes the normally membrane-bound protein to be secreted instead (Falivelli et al., 2012), presumably eliminating its normal functionality, and possibly introducing novel effects.. Another mutant, D1129H (Figure 2), also prevents surface expression of CNTNAP2, and instead the protein remains restricted to the endoplasmic reticulum, unable to move to the plasma membrane, interferes with the LNS4 domain of CNTNAP2, and is presumed to cause misfolding of the protein. Most other mutations investigated did not show restricted localization to the ER, though a mutation in a highly conserved amino acid, I869T (Figure 2), had less surface staining than the wild type form of the protein. Theoretically, mutations that interfere with intracellular trafficking of CNTNAP2 would also interfere with protein function. However, with the exception of 3709delG, these mutations do not always result in an autistic phenotype, indicating that other genetic, environmental, and developmental factors are involved in the presentation of the disorder.

CNTNAP2 Function in the Brain

Investigation of genes related to the formation of language-related brain areas revealed *CNTNAP2* enrichment in the cortical superior temporal gyrus, associated with language processing and production (Abrahams & Geschwind, 2008). Moreover, *CNTNAP2* is enriched in embryonic human frontal cortex, but not in rat or mouse at comparable stages of development. Not only do these data suggest a potential role for CNTNAP2 in the development of neural circuitry underlying language, they conform to the idea that this enrichment is relevant to vocal learning in humans, a behavior not shared with rodents.

The brains of healthy and autistic individuals homozygous for risk alleles rs7794745 and rs2710102 exhibit functional differences. Subjects with one or both risk variants exhibit increased activation of the frontal operculum and medial frontal gyrus relative to subjects homozygous for the

Figure 2. Schematic of human CNTNAP2. CNTNAP2 consists of a single discoidin domain (DISC), four laminin-G domains (LamG), EGF repeats, a single transmembrane region (TM), and a putative protein 4.1 binding region (4.1m). CDFE indicates the subregion of the protein that is deleted in cases of cortical dysplasia-focal epilepsy in an Old Order Amish population (Li et al., 2004; Strauss et al., 2006). Arrows indicate two other amino acid changes associated with language impairment (869 and 1129).

non-risk allele (Whalley et al., 2011). Event-related brain potentials are altered during a language perception task in individuals carrying the rs7794745 risk allele (Kos et al., 2012). Scott-Van Zeeland, Abrahams, et al. (2010) investigated the correlation of risk allele rs2710102 with connectivity both within the medial prefrontal cortex (mPFC) and between other areas. In this study, subjects participated in a reward-based learning task in which they were presented with abstract images and were asked to assign them into either "Group 1" or "Group 2." Upon correct classification, subjects were either given a monetary or social reward, or a "neutral" reward in which they were simply told whether or not they were correct. This experimental paradigm activates frontostriatal circuits (Scott-Van Zeeland, Dapretto, Ghahremani, Poldrack, & Bookheimer, 2010). Subjects with the CNTNAP2 risk allele rs2710102 exhibited increased local connectivity in the mPFC relative to subjects without the risk variant. This occurred in a genetically dominant fashion regardless of the autism phenotype of the risk allele carriers. In addition, risk allele carriers had less focused long-range connectivity between the mPFC and several other brain areas, as well as decreased lateralization, a result which is associated with autism-like behaviors. These data suggest that CNTNAP2 variants increase the risk of autism through alteration of frontal lobar connectivity.

Animal Models for Cntnap2

As yet, the most well-characterized function of Cntnap2 is to cluster voltage-gated potassium channels at juxtaparanodes of axons in the peripheral nervous system (Poliak et al., 2003). Recently, another potential function was discovered through an RNA interference (RNAi) survey of autism susceptibility genes (Anderson et al., 2012). Of the 13 genes included in the RNAi screen, Cntnap2 knockdown had the most pronounced effects on network activity in mouse hippocampal cultures. In mouse cortical cultures transfected with short hairpin RNA (shRNA) targeting endogenous Cntnap2, calcium transients from evoked synaptic responses were reduced in amplitude to approximately 70% of controls, though action potential frequency was not affected. Conversely, knockdown of the binding partner of Cntnap2, contactin 2, had the opposite effect, increasing the amplitude of the action potential. Cntnap2 expression level has no effect on neuronal excitability. Instead, the underlying cause of the action potential attenuation is a global decrease in synaptic transmission. Both excitatory and inhibitory evoked currents are reduced by the shRNA, as well as the frequency of miniature postsynaptic potentials, suggesting that the number of synaptic sites on affected neurons is reduced. This is further confirmed by changes to cellular morphology of transfected neurons. Cntnap2 knockdown results in shorter neurites with fewer branches, and dendritic spines with smaller spine heads. These data are evidence that Cntnap2 may affect the development of neurons by increasing the number of active synaptic sites and facilitating network activity.

Given the evidence for a role of *CNTNAP2* in human speech, it may also function in birdsong (Panaitof, Abrahams, Dong, Geschwind, & White, 2010). In adult male zebra finches, *Cntnap2* transcript is enriched in the robust nucleus of the arcopallium (RA) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN), cortical nuclei in the song production system. Projection neurons from RA are similar to layer 5 pyramidal neurons in mammalian cortex whose axons descend below the telencephalon to synapse onto motor neurons (Jarvis, 2004), and LMAN shares similarities with the mammalian prefrontal cortex (Kojima, Kao, & Doupe, 2013). No such enrichment of *Cntnap2* is observed in HVC (acronym used as a proper name), another song nucleus analogous to mammalian cortical layer 2/3 (Jarvis, 2004), and there is reduced expression in area X relative to the striatopallidum. Each song nucleus is comprised of similar cell types as those in the surrounding tissues, which suggests that the differential expression of genes within the song nucleus indicates a specific role for those genes in vocal learning and/or production. In contrast to males, adult females have moderate transcript levels in RA and LMAN. Female zebra finches have an underdeveloped area X that is not visible by common staining procedures (Balmer, Carels, Frisch, & Nick, 2009), but still *Cntnap2* is uniform across the entire striatopallidum. Interestingly, in young females (<50d) *Cntnap2* is enriched in RA to the same degree as for males, and declines to the level of the surrounding arcopallium with age. The reduction in gene expression coincides with the sensorimotor period of song learning in males, a time at which the male begins to practice singing. The percentage of cells expressing the protein in female RA decreases at this time point (Condro & White, 2014). This sexually dimorphic expression supports the hypothesis that *Cntnap2* expression in RA is important for proper production of learned vocalizations in songbirds. According to this hypothesis, interference of Cntnap2 translation in male RA should disrupt song learning and/or production (Haesler et al., 2007).

As with Foxp2, mouse models of Cntnap2 risk variants may not capture language deficits associated with their respective disorders. However, they can be used to study other aspects of behavior and physiology that may impact future studies focused on vocal learning. Initially, outbred Cntnap2(-/-) mice were reported to have no gross anatomical or neurological abnormalities (Poliak et al., 2003). However, when these mice were crossbred with the C57BL/6J strain,

subsequent generations exhibited neurological abnormalities similar to human patients with CDFE (Strauss et al., 2006), including epileptic seizures induced by mild handling starting before 6 months of age (Penagarikano et al., 2011). These knockout mice present neuronal migration abnormalities, with an increase in the incidence of ectopic neurons, a reduced number of inhibitory interneurons in the cortex and the striatum, along with impaired network synchrony in the cortex. Additionally, there is increased spontaneous inhibitory activity in cortical layers 2/3, disrupting the balance between inhibition and excitation (Lazaro, Penagarikano, Dong, Geschwind, & Golshani, 2012). These mice exhibit behavior similar to the human autistic phenotype, including repetitive motions, such as self-grooming and digging, behavioral inflexibility on learned tasks, such as the Morris water maze or T maze, decreased social activity with other mice, reduced nest building, and a decrease in the number of ultrasonic separation calls. Less frequent vocalizations could be symptomatic of impaired communication similar to language regression in autism, or alternatively due to a decreased motivation for maternal interactions, similar to social impairment in autistic children. The two hypotheses are not mutually exclusive, though the former is less likely, since this particular call type in mice is innate (Arriaga et al., 2012) and therefore not subject to regression. Interestingly, many of the behavioral deficits in the knockout mice can be partially rescued by treatment with risperidone, a medication used to treat the symptoms of autism (Penagarikano et al., 2011). However, the drug does not improve social interactions for the knockout mice. The effects of risperidone on communicative behavior have not yet been reported. Rescue by the drug of some of the effects of knocking out *Cntnap2* further validates the relationship between *Cntnap2* and autism. These knockout mice can be used to test other drugs to treat some of the symptoms of autism, though perhaps not language impairment. This model is especially pertinent to CDFE, for which the mutation renders CNTNAP2 nonfunctional. The more common polymorphisms associated with ASD and SLI risk lie in introns, creating a challenge to develop mouse models. A songbird model may offer an advantage in understanding the role of CNTNAP2 in language in that knockdown of Cntnap2 can be targeted to song nuclei, isolating its effects on vocal behavior.

4. Hepatocyte Growth Factor Signaling Pathway Genes

In keeping with the theme of FoxP2 as a molecular entry point into gene networks involved in speech and language, another class of FoxP2 target genes is implicated in language deficits. Three genes in the hepatocyte growth factor (HGF) signaling pathway are each targets of FOXP2 regulation and associated with disorders of human speech and language. The first is the HGF receptor tyrosine kinase MET (Bottaro et al., 1991), which has been linked to ASD (Mukamel et al., 2011). The second, also linked to ASD, is the urokinase plasminogen activator receptor (uPAR, or *PLAUR* when referring to the human gene; Campbell et al., 2007), which was long thought to indirectly activate HGF through its binding partner urokinase (Mars, Zarnegar, & Michalopoulos, 1993), though more recently this function has been challenged (Eagleson, Campbell, Thompson, Bergman, & Levitt, 2011; Owen et al., 2010). The third is sushi-repeat protein, X-linked 2 (SRPX2), a uPAR ligand (Royer-Zemmour et al., 2008) that also binds HGF (Tanaka et al., 2012), and may account for the HGF-mediated effects of uPAR signaling. SRPX2 is linked to language through association with childhood seizures of the Rolandic fissure, which can cause language disabilities (Roll et al., 2006). FOXP2 binds the promoter regions of all three genes and represses transcription (G. Konopka et al., 2012; Mukamel et al., 2011; Roll et al., 2010). Recent evidence suggests that FOXP2 regulation of SRPX2 affects synaptogenesis and vocalizations in mice (Sia, Clem, & Huganir, 2013). Similar to *CNTNAP2*, the distribution of *MET* in human fetal brain is complementary to that of *FOXP2*. In cultures of normal human neural progenitors and established cell lines, endogenous *FOXP2* expression increases with maturity as *MET* decreases (G. Konopka et al., 2012). Notably, the KE mutant (R553H) fails to repress *uPAR* or *SRPX2* (Roll et al., 2010). These data suggest that HGF signaling is altered in cases of language disorders associated with FOXP2. To date HGF itself has not been directly associated with a disorder relating to speech; however, given the association of these other HGF signaling pathway genes with language disorders, it would not be surprising if such an association were discovered.

MET was initially investigated as an autism susceptibility gene due to the similarity of neuroanatomical abnormalities attributed to loss of MET signaling in the cortex and those found in cases of autism (Campbell et al., 2006). A SNP in the promoter region of *MET*, rs1858830, was identified as a site associated with elevated risk of diagnosis of autism. The "C" variant at this site causes a reduction in transcription of the gene, and alters transcription factor binding relative to the non-risk "G" variant. The "C" variant is overrepresented in cases of ASD, associated with reduced MET protein in the cortex (Campbell et al., 2007; Campbell, Li, Sutcliffe, Persico, & Levitt, 2008) and social and communication impairments in cases of ASD (Campbell, Warren, Sutcliffe, Lee, & Levitt, 2010). In healthy human embryonic brains, MET is enriched in the temporal cortex, an area involved in language processing,

and to a lesser degree in the hippocampus and occipital cortex (Mukamel et al., 2011). HGF signaling through MET promotes development of cortical projection neurons (Eagleson et al., 2011). In microarray analysis, *MET* has been identified as a member of a gene module correlated with differentiation, particularly with axon guidance (G. Konopka et al., 2012). Though protein levels are dynamic during development, a peak of expression coincides with increased development of neurites and synapse formation, suggesting a role for MET in neuronal connectivity (Judson, Bergman, Campbell, Eagleson, & Levitt, 2009). MET is expressed in axon tracts of projection neurons of the neocortex, including those that descend into the striatum, consistent with the hypothesis that MET is a factor in development of neural circuits, which when perturbed, leads to symptoms of ASD and language impairment.

In a screen for other ASD-related genes in the MET signaling pathway, a SNP in the promoter region of *PLAUR*, rs344781, was identified as a risk factor for autism diagnosis with an interaction effect with *MET* rs1858830. *uPAR* knockout mice have been generated, but thus far studies have focused on the effects of knockout on neural migration and seizure activity. Whereas MET seems to promote cortical projection neuron migration and growth, uPAR seems to affect inhibitory neurons in much the same manner, though the mechanism remains unclear (Eagleson et al., 2011). Homozygous knockouts exhibit spontaneous seizures as well as a reduction of parvalbumin-positive interneurons in the anterior cingulate and parietal cortices (Eagleson, Bonnin, & Levitt, 2005; Powell et al., 2003). The loss of inhibitory interneurons may affect the balance of excitation and inhibition, a phenomenon associated with autism (Eagleson et al., 2011). Interestingly, *uPAR* may be absent in birds (NCBI search, BLAST), suggesting that it is not common to all vocal learning species. There may be a different molecule in songbirds that replaces uPAR function. Though uPAR was originally thought to be involved in the activation of HGF required for binding to MET (Mars et al., 1993), recent evidence suggests that uPAR and its binding partner urokinase contribute very little to the process, and rather other serine proteases are responsible for HGF activation (Owen et al., 2010). Phenotypic differences in *uPAR* and *MET* knockout mice support this hypothesis (Eagleson et al., 2011). However, uPAR is involved in several signaling cascades independent of MET (Blasi & Carmeliet, 2002), any of which may be related to autism or language impairment.

SRPX2 is a chondroitin sulfate proteoglycan that binds to both HGF and uPAR (Royer-Zemmour et al., 2008; Tanaka et al., 2012). Mutations in SRPX2 can result in seizures originating in the Rolandic fissure, which can lead to abnormal brain morphology in the form of polymicrogyria, and are associated with oral and speech dyspraxia and cognitive impairment (Roll et al., 2006). One such mutation, resulting in a tyrosine-to-serine substitution at position 72, is related to both Rolandic seizures and orofacial and fine motor impairment. The substitution occurs in a region thought to affect protein–protein interactions. In this same region, a site at position 75 is highly conserved among primates, but has changed in humans since the split from chimpanzees, suggesting an evolutionary mechanism for human speech (Royer et al., 2007), reminiscent of the amino acid substitutions in FOXP2 between the two species (Enard et al., 2002). As mentioned previously, new evidence has emerged for the role of SPRX2 in mouse vocalizations (Sia, Clem, & Huganir, 2013). Other chondroitin sulfate proteoglycans are involved in formation of perineuronal nets, which can affect plasticity of sensory systems (McRae, Rocco, Kelly, Brumberg, & Matthews, 2007). In songbirds, development of perineuronal nets around song nuclei correlates with the development of song, and it is hypothesized that destruction of these nets permits the reopening of critical period for song learning after crystallization (Balmer et al., 2009). It is possible, therefore, that SRPX2 is involved in similar processes, which could affect learned vocalizations in humans and songbirds alike.

5. Stuttering Genes

Stuttering, or stammering, is a condition in which speech is interrupted by involuntary repetitions of syllables or words, prolongation of syllables, or pauses during speech. Inheritance patterns strongly suggest a multifactorial genetic basis for the disorder, with relatively little environmental influence (Kang et al., 2011; Kraft & Yairi, 2012). However, it was not until recently that any specific gene was identified as a factor in stuttering. Genome-wide linkage revealed a locus of disequilibrium on chromosome 12 for stuttering (Riaz et al., 2005), which was investigated more closely in a large pedigree, identified only as Family PKST72, in which roughly half of the living members stutter (Kang et al., 2010). Genotyping in this pedigree revealed a relationship with a SNP, (G3598A), which causes a glutamine-to-lysine amino acid substitution in a gene encoding a subunit of *N*-acetylglucosamine-1-phosphate transferase (*GNPTAB*). The ‘A’ variant was more common in stuttering family members, and family members homozygous for the ‘G’ variant were much less likely to stutter. Unlike *FOXP2* in the KE family, though, G3598A exhibits some phenotypic plasticity, in that not every family member with an ‘A’ variant stutters, and some family members homozygous for the ‘G’ variant do stutter. Sex has been previously shown to be a factor in recovery of stuttering, with females being four times more likely to recover (Ambrose, Cox, & Yairi,

1997). Such may be the case for the two female non-stuttering family members homozygous for the 'A' variant (Kang et al., 2010). Three more amino acid changes in GNPTAB were associated with stuttering in a broader population sample, as well as three others found in GNPTG, another subunit of the phosphotransferase, and three more mutations in *N*-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase (NAGPA). These mutations account for a small percentage (<10%) of stuttering cases in this study, indicating that still unidentified factors contribute to the disorder. GNPTAB, GNPTG and NAGPA act as enzymes in the lysosomal targeting pathway. Other mutations in GNPTAB and GNPTG are associated with mucolipidoses, disorders associated with deficits in development, mental ability, and speech, though this study is the first to link mutations in these genes to stuttering (Kang et al., 2010; Kang & Drayna, 2012). The mechanisms by which these mutations affect speech are unknown. Other loci have been identified as potential sites for mutations associated with stuttering (Kraft & Yairi, 2012; Raza, Amjad, Riazuddin, & Drayna, 2012), but as yet no other genes have been discovered. One study did find an association between a SNP in the *DRD2* gene in a Chinese Han population (Lan et al., 2009), but this result was not replicated in a larger sample (Kang et al., 2011). Additionally, a case was reported in which a partial deletion of CNTNAP2 was found in a stuttering patient, (Petrin et al., 2010) suggesting that there may be some overlap of genetic factors in stuttering and other language disorders.

6. Other Genes of Interest

Additional genes likely contribute to vocal learning. In a screen of genes within a region on chromosome 16 associated with SLI, two candidates correlated with measures of language ability: c-maf-inducing protein (CMIP) and calcium-importing ATPase, type 2C, member 2 (ATP2C2; Newbury et al., 2009). A subsequent study found an association of CMIP, but not of ATP2C2, with reading-related measures (Newbury et al., 2011; Scerri et al., 2011). Though both molecules are expressed in the brain, their functions therein are still poorly understood. In other tissues, CMIP is involved in a cell signaling cascade (Grimbert et al., 2003), and ATP2C2 is part of a pathway responsible for shuttling divalent ions to the Golgi apparatus (Faddy et al., 2008; Missiaen, Dode, Vanoevelen, Raeymaekers, & Wuytack, 2007). Other genes potentially involved in language comprehension include doublecortin domain containing protein 2 (*DCDC2*) and *KIAA0319*, which have both been associated with dyslexia (Czamara et al., 2011; Newbury et al., 2011; M. L. Rice, Smith, & Gayán, 2009; Scerri et al., 2011). Recently, DCDC2 was found to affect neuronal firing, increasing the excitability and compromising spike timing (Che, Girgenti, & Loturco, 2013). Given that the other genes implicated in language acquisition and production seem to be involved in either neurogenesis or neurite growth, perhaps CMIP, ATP2C2, and DCDC2 affect either or both of these processes. However, the function of KIAA0319 in language processing is beginning to be better understood. KIAA0319 is involved in the clathrin endocytosis pathway (Levecque, Velayos-Baeza, Holloway, & Monaco, 2009). Knockdown of Kiaa0319 expression in rat auditory cortex results in increased neuronal input resistance accompanied by increased excitability in response to auditory stimuli (Centanni et al., 2013). The authors hypothesize that this change in neuronal excitability, relevant to variants of KIAA0319 in cases of dyslexia, impedes differentiation of speech and non-speech sounds. Another gene of interest in relation to its role in language is *FMR1*, which encodes the fragile X mental retardation protein (FMRP). Language delay and impairments in both receptive and expressive language are characteristic of children with fragile X syndrome (FXS; Finestack, Richmond, & Abbeduto, 2009). In the zebra finch song system, FMRP is expressed in song nuclei HVC, LMAN, RA, and area X (Winograd, Clayton, & Ceman, 2008). Interestingly, FMRP is enriched in male RA around the onset of the sensorimotor learning phase. These data suggest that FMRP may be a common factor in learned vocalizations in both humans and songbirds.

7. MicroRNA

MicroRNAs (miRs) are short (~22 nucleotide), noncoding RNAs that post-transcriptionally regulate synthesis of specific proteins through either degradation of the mRNA or inhibition of translation (He & Hannon, 2004; Pasquinelli, 2012). These small molecules are thought to "fine-tune" gene expression involved in many biological processes. Research on miR functions in the brain has focused primarily on roles in development and neurogenesis (Liu & Zhao, 2009; Sun, Crabtree, & Yoo, 2013), though studies are starting to emerge on activational effects in the mature brain (Bredy, Lin, Wei, Baker-Andresen, & Mattick, 2011; Fiore, Khudayberdiev, Saba, & Schratt, 2011; Shi et al., 2013). MicroRNAs can affect learning and memory-based tasks, such as fear conditioning, context conditioning, place preference, and Morris water maze performance (Griggs, Young, Rumbaugh, & Miller, 2013; Konopka et al., 2010; Olde Loohuis et al., 2011; Wang & Barres, 2012). Another class of small noncoding RNAs are those that interact with regulatory piwi proteins (piRNAs) in spermatogenic cells, whose mechanisms and functions are still poorly understood, though evidence suggests they are involved in epigenetic control of transcription (Kuramochi-Miyagawa et al., 2008). Recently, piRNAs

have been identified as factors contributing to associative learning in *Aplysia* through regulation of CREB2 (Rajasethupathy et al., 2012). However, investigation into the role of small RNAs in vocal learning has only just begun.

As with many of the genes described in this review, *FOXP2* may be used as a starting point by identifying miRs that regulate expression of FOXP2, or are targets of FOXP2 regulation (or in some cases, both). In microarray analysis used to identify gene networks influenced by Foxp2 expression, 22 miRs were identified as transcriptional targets of murine Foxp2 (Vernes et al., 2011). Of these, several have documented functions in the brain: miR-9, -29a, -30a, -30d, -34b, -124a, -125b, and -137. Additional sources of potential vocal learning–associated miRs come from studies in songbirds. In zebra finches, miR-137 was included in a microarray study investigating genes regulated by singing in basal ganglia nucleus area X, and was found to belong to the same gene network module as *FoxP2*, and negatively associated with the number of motifs sung (Hilliard, Miller, Fraley, et al., 2012). As mentioned in an earlier section, miR-9 and -140-5p are expressed in zebra finch area X, are upregulated by singing in juveniles and adults, and associated with reduced levels of *FoxP2* mRNA (Shi et al., 2013). Expression of five miRs in cortical auditory regions are affected by exposure to conspecific song: mir-92, -124, and -129-5p decreased, and mir-25 and -192 increased (Gunaratne et al., 2011). Though the birds in this latter study were adults, and therefore past the critical phase of song learning, the miRs involved in auditory processing may very well impact song learning earlier in life. mir-2954, a putatively avian-specific miR, is expressed at greater levels in males than females in all tissues tested, including brain (Luo et al., 2012). miR-2954 may therefore play a role in the sex-based differences in neuroanatomy and song learning in this species. miRs like miR-2954, which appear to be unique to birds or specifically zebra finch (Gunaratne et al., 2011; Luo et al., 2012), are not likely a common factor underlying behavior in all vocal learning species, although they may regulate genes in a manner common to all vocal learners. A better understanding of the mRNA targets of these miRs will be required to parse out this hypothesis.

How might miRs in the brain affect vocal learning? As with other genes implicated in vocal learning, many miRs act early in development to regulate neurogenesis (Sun et al., 2013), which may contribute to the organization of brain structures underlying speech and vocal learning. In chick spinal cord, miR-9 acts through regulation of FoxP1 to direct motor neuron specification (Otaegi, Pollock, Hong, & Sun, 2011). In the ventricular zone of developing mouse and zebra fish brain, miR-9 promotes neural differentiation by suppression of proteins involved in the proliferation of neural stem cells (Coolen, Thieffry, Drivenes, Becker, & Bally-Cuif, 2012; Saunders et al., 2010; Shibata, Nakao, Kiyonari, Abe, & Aizawa, 2011; Tan, Ohtsuka, González, & Kageyama, 2012; Zhao, Sun, Li, & Shi, 2009). Similarly, miR-124 expression in the developing CNS is thought to direct cell differentiation to a neuronal fate by suppressing non-neuronal transcripts (Cheng, Pastrana, Tavazoie, & Doetsch, 2009; Lim et al., 2005; Makeyev, Zhang, Carrasco, & Maniatis, 2007; Sanuki et al., 2011; Visvanathan, Lee, Lee, Lee, & Lee, 2007). miR-137 also regulates maturation of neurons (Smrt et al., 2010).

Additionally, miRs may have activational effects that support vocal learning. Several miRNAs impact neurite outgrowth and synaptogenesis. miR-9, for example, is expressed in axons of post-mitotic cortical neurons and limits or fine-tunes axon growth (Dajas-Bailador et al., 2012). Brain-derived neurotrophic factor (BDNF) indirectly affects axon growth through regulation of miR-9. Application of BDNF for a short period reduces miR-9 levels and subsequent growth of the axon, but prolonged exposure leads to an increase in miR-9 and a cessation of axon growth. In the songbird, BDNF is thought to be an important factor for neural connectivity between motor song nuclei in development and in adulthood in seasonal learners (Brenowitz, 2013); therefore, miR-9 activity in the songbird brain may be regulated by BDNF exposure. Additionally, predicted binding sites for miR-9 are found in the 3'-untranslated region of matrix metallopeptidase-9 (*MMP9*), an enzyme that affects synaptic morphology (Konopka et al., 2010). miR-9 represses both *Foxp1* (Otaegi et al., 2011) and *Foxp2* (Clovis et al., 2012; Shi et al., 2013), whereas Foxp2 promotes miR-9 expression in neuron-like cells in culture (Vernes et al., 2011). This argues for the existence of a Foxp2/miR-9 feedback loop, in which miR-9 indirectly affects gene expression downstream of FoxP2. miR-29a/b changes dendritic spine morphology in hippocampus (Lippi et al., 2011). In *Aplysia*, miR-124 restricts serotonin-induced synaptic plasticity through regulation of CREB (Rajasethupathy et al., 2009). In mouse differentiating and adult primary cortical neurons, overexpression of miR-124 increases neurite outgrowth, whereas functional blockade causes a delay (Yu, Chung, Deo, Thompson, & Turner, 2008). miRs may affect synaptic plasticity by regulating synaptic molecules. miR-137 has potential binding sites in the 3'UTR of *GluR1* mRNA, and miR-124 in *GluR2* (Konopka et al., 2010). Regulation of these proteins could impact the synaptic plasticity required for vocal learning.

Conclusions

Recent advances have augmented our understanding of the genetic basis for vocal learning by (a) uncovering

new genetic factors through studies of human pathology, (b) discovering new vocal learning–related genes through network analysis of neural tissues pertaining to human speech and birdsong, and (c) developing a better understanding of the physiological effects of known speech-related genes, such as *FOXP1*, *FOXP2*, and *CNTNAP2* using animal models. *FOXP2* was the first gene directly correlated with a language disorder, and through its molecular connections other language-related genes are being discovered, including those in the HGF signaling pathway. As small RNA regulatory factors become better cataloged, we are likely to learn even more about the genetic basis of vocal learning. Since convergent evolution has produced vocal learning in humans, other mammals, and songbirds, we might expect that there are overlapping genes between the clades, but equally we expect some differences. This is likely the case with uPAR, which has no direct avian correlate, but is associated with human speech pathology. Continuing investigation into genes that affect language and vocal learning in other species will provide a better understanding of the mechanisms that govern this complex communicative behavior.

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FoxP2 and vocalization

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From a linguist's point of view, the ability to vocalize new sounds may not seem to be a critical component of language. Yet when this ability is impaired, the social and emotional consequences for the affected individual can be severe, as evidenced by those suffering from developmental or injury-related speech disorders. How are we to understand this vocal learning trait, and where should it be placed within a framework for language evolution? Here, I argue that studying the supporting brain pathways that are affected in vocal learning disorders is a good place to start. Since such study is largely limited to noninvasive methods in humans, investigating other animals that possess this rare trait paves the way for a comparative analysis of the molecular, cellular, and synaptic bases of vocal production learning, including human speech. This kind of inquiry can highlight shared evolutionary pathways as well as key detours.

1. Introduction

Not only is language unique to humans, language is unique. No other behavior exhibits the same suite of seemingly conflicting features, including being innately predisposed yet highly dependent on social experience; requiring practice yet remaining unstereotyped; amenable to rapid-fire interchange yet infinitely expressive. When language is (artificially) deconstructed into separate subcomponents, some similarities to other behaviors emerge. In this chapter, I focus on vocal production learning. This subcomponent can be likened to the fine art of learning to draw or paint. Both skills arise through what scientists call "procedural learning," to distinguish them from "declarative learning." The latter can be accomplished through the verbal or written transfer of information. In contrast, no one can tell you how to ride a bike or how to produce a new word or painting. Rather, these acts depend on trial-and-error learning along the lines of "practice makes perfect." Both rely on sensory input to guide them and on feedback to perfect them. In the case of vocal learning, the main (but not sole) sensory domain is hearing, whereas in painting, vision is key. In both, heightened control of the muscles that participate in creating the new sound or image must be trained. Once such control is mastered, the creative possibilities abound for the skilled speaker or artist.

Regardless of whether or when vocal learning arose as a bona fide subcomponent in the evolution of human language, it can be argued, as Nottebohm has, that once this ability was in place, it enabled the development of "an open-ended system of sounds that can be used… for the further development of language" (Nottebohm & Liu 2010, p. 3). Thus, while not the most unique subcomponent of language, vocal learning in the hominid lineage could have supported and/or reinforced language evolution. Investigation of how the brain accomplishes this special sensorimotor skill, which is shared among only a handful of animal groups, has already revealed a surprising number of developmental, anatomical, and molecular commonalities.

2. Vocal production learning: What is it, who does it, and how do you know?

How can one prove that a given species, such as *Homo sapiens*, is innately capable of producing its vocal communication signals rather than having to learn them? What would be a definitive experiment? "Take some disenfranchised children off to an island and raise them without talking with them," you say? Though shocking, Akbar the Great (1542–1605) did just that (see Cohen, this volume). As the third Mughal emperor, he commanded that the infants be reared by mute nuns and then, at 12 years of age, be returned to court for analysis. There, the children failed to make any decipherable utterances, even though Akbar had astutely convened judges from many lands in order to detect any rare languages that the children might have produced. While today such experiments are legally and morally prohibited, they serve to illustrate one general approach for gathering such proof in nonhuman animals.

In modern times, a new group of animals has made the list of vocal production learners (Poole et al. 2005). In this case, careful acoustic analysis has overcome the limitations of studying a surprisingly low number of subjects, namely two. These two were Mlaika and Calimero, African elephants living in captivity, where they were noticed to produce atypical sounds. Scientists compared their vocalizations to those of other elephants, including Asian ones, and to other noises. Although Mlaika made some "normal" sounds that overlapped in length and frequency (perceived as pitch) with other African elephants' sounds, she also uttered a distinct set of longer and higher vocalizations that overlapped with the sounds made by trucks, recorded from the nearby highway. Calimero's vocalizations, on the other hand, were intermediate in length and frequency between those of his own species and those typical of Asian elephants, with whom he had been housed as a juvenile. Together, the stories of Akbar and the elephants illustrate the types of experiments that test for vocal production learning. Animals genetically endowed with this ability but that are raised in impoverished environments, where they are deprived of hearing their own species, fail

to develop normal vocalizations and/or imitate uncharacteristic but more abundant sounds which they are not hard-wired to produce.

A more severe test of the innateness of a species' vocalizations is the deprivation of all sounds that occurs with deafness. Deafness early in development is the most devastating because it delivers two blows to vocal learning: (1) it eliminates the imitative model by preventing the learner from hearing others of its species. Equally devastating, (2) the learner cannot hear his or her own vocalizations, preventing the auditory feedback necessary for vocal imitation and refinement. Loss of hearing in adulthood causes more subtle speech deficits that accumulate over time by preventing the speaker from continuously monitoring and updating his/her speech quality. Although we are not readily conscious of such monitoring, it can be experimentally revealed in adults with normal hearing by using headphones to deliver playback of their speech while they are speaking. If the speech is played back with a slight delay it can artificially induce the speaker to stutter (Lee 1950).

Due to practical considerations, not all animals have been rigorously tested for vocal production learning, but many nonhuman primates have. Our closest relative, the chimpanzee (*Pan troglodytes*), from whom we diverged some 6 million years ago, shares 95% of our DNA sequences (this number jumps to 99% for gene coding regions; more on this topic below), yet none of our vocal learning capacity (Pollard 2009). For example, a young female chimpanzee named Vicky was raised by her keepers in their home as if she were a human child. After six and a half years of training, she was only capable of uttering the distinguishable words *mama, papa,* and *cup* (Wallman 1992). This abysmal level of verbal output does not mean that chimpanzees are incapable of understanding language. Indeed, Vicky and additional chimpanzee subjects exhibit significant language comprehension (Terrace et al. 1979). And today, the bonobo (*Pan paniscus*) known as Kanzi demonstrates an impressive ability to both understand and "talk" with human caretakers when trained to point to pictograms in order to express himself, rather than to vocalize (see Savage-Rumbaugh, this volume). It is just that, based on their peripheral and central anatomy, chimpanzees and other nonhuman primates lack the physical capacity for developing the specialized control of the muscles necessary for noninnate vocal output. Along the vocal tract, these include the larynx, pharynx, tongue, teeth, and lips, as well as the muscles of respiration. All of these muscles are controlled by motoneurons in distinct regions of the brainstem. When the motoneurons fire, the muscles that they contact contract. What appears crucial is the next step back in the pathway leading to the motoneurons. In humans, but not in nonhuman primates, neurons in the motor cortex directly innervate laryngeal motoneurons. This neural connectivity or "wiring pattern" appears necessary for producing learned vocalizations, but is dispensable for innate vocal patterns, which depend upon a separate pathway (Jurgens 2009). Whether elephants possess the

crucial direct connection between motor cortex and motoneurons in their vocal control pathway is as yet unknown.

In addition to humans and elephants, the short list of animals demonstrated to be vocal production learners is currently limited to songbirds, parrots, and hummingbirds, which are in separate taxonomic orders (raising the hypothesis that the trait emerged independently three times in the avian lineage), and certain species of marine mammals and bats. Of these, the learned song produced by songbirds is the best characterized and exhibits significant parallels to human speech (Jarvis 2004). Shared features include the facts that both are learned through social interactions with conspecifics, both occur naturally and spontaneously within the organism's own species-characteristic behavior, and, as outlined above for humans, both depend upon auditory experience. As will be detailed below, learned birdsong also shares developmental, anatomic, and genetic components with speech.

With the goal of discovering the biological bases for vocal production learning and relating these to language evolution, an important advantage of certain songbird species is that they readily breed in the laboratory where they can be reared under controlled experimental conditions. Moreover, the brain pathways that support song learning and production are easily identified, especially in species such as the zebra finch in which only males learn to sing (i.e. their courtship songs, which are then listened to and selected for by female zebra finches). Congruent with the sexually dimorphic behavior in this species, the underlying neuroanatomical pathways are also sexually dimorphic (Nottebohm & Arnold 1976). Only males possess the full suite of interconnected brain regions that support song. These structures are dedicated to song learning and production, presenting excellent targets for the manipulation of brain circuits related to vocal learning without disrupting other cognitive processes. This is not the case in humans, nor in other vocal learning species studied thus far. Due to these unique features, songbirds such as the zebra finch provide an advantageous animal model to identify the molecular, cellular, and synaptic bases for vocal production learning.

2.1 Parallel vocal developmental programs

Similar to humans, songbirds learn their vocalizations best early in development. Learning involves two critical periods that can be distinguished by the source of the auditory input required for normal development. In the first critical period, termed "sensory acquisition," young songbirds listen to and memorize the song of an adult tutor. In zebra finches, sensory acquisition begins around the time of fledging (~20 days post-hatching) and ends by 65 days (Immelmann 1969), at which time a normally reared finch will become refractory to learning additional songs (White 2001). A second critical period known as "sensorimotor learning" occurs when young

birds begin to produce new sounds and to use auditory feedback of their own vocalizations to perfect a match to the memorized model. The onset of this process has been likened to human infant babbling (Doupe & Kuhl 1999). As sensorimotor learning progresses, the previously rambling and variable song becomes increasingly stereotyped such that by sexual maturation, which occurs at ~100 days in zebra finches, the song is sung relatively unchanged throughout adulthood.

The stereotyped nature of adult zebra finch song appears to contrast with the less limited capacity of human vocalizations. However, a broader comparison of vocal learning in the >4,000 species of songbirds to human speech reveals shared developmental constraints, as well as relative openness to experiential input throughout life, coupled with ongoing dependence on hearing. Specifically, the degree of vocal flexibility in mature songbirds varies with the species. Mockingbirds, for example, are capable of learning new songs throughout their lives. Even in zebra finches, mature song is not fixed but rather requires continuous auditory feedback in order to be maintained, as described above for human speech. The so-called "crystallized" song of zebra finches nonetheless deteriorates in birds deafened in adulthood (Brainard & Doupe 2000a; Nordeen & Nordeen 1992). Also like speech, mature birdsong can be disrupted in normal hearing birds exposed to abnormal auditory feedback (Andalman & Fee 2009; Cynx & Von Rad 2001; Sober & Brainard 2009). On the human side, although there are clearly some "mockingbirds" among us, the ability to learn new languages without an accent is generally best accomplished prior to puberty (Doupe & Kuhl 1999).

2.2 Anatomical parallels

In 2004, the cell groups and fiber tracts of the avian brain were renamed in accordance with data that had accumulated prior to and since the publication of the stereotaxic atlas of the pigeon brain (Karten & Hodos 1967; Reiner et al. 2004). The new nomenclature corrects previous erroneous assumptions about the origin of avian neural tissue and the limitations of avian intelligence, and reinforces the similarity between avian and mammalian circuits. As a result, birds, including songbirds, are now acknowledged to possess a substantial amount of cortex, in addition to basal ganglia. The basal ganglia were previously thought to form the bulk of the avian telencephalon and to account for the overly instinctual behaviors of birds – another erroneous assumption. Along with a substantial cortex, certain avian species are now recognized to possess more sophisticated cognitive capacities than those exhibited by the domesticated, flightless, non-vocal-learning chicken, most familiar to humans. Even the microcircuitry within the primary avian auditory cortex has been found to comprise radial columnar arrays virtually identical to those of the mammalian auditory cortex (Wang et al. 2010).

Within the brains of songbirds, but not in non-vocal-learning birds, distinct subregions of the cortex, basal ganglia, and thalamus are dedicated to song learning

and production. Outside of these subregions, the cell types are similar to those found within, but the functions of the neurons are diverse and ill defined. This special feature whereby neurons dedicated to vocal production learning are grouped together within a given brain region, greatly facilitating their anatomical and functional identification, thus far appears limited to avian vocal learners. In songbirds, these brain regions and their interconnections are collectively referred to as the song circuit.

The song circuit consists of two component pathways: a vocal motor backbone, referred to as the posterior vocal pathway (in the back of the brain), and the anterior forebrain pathway (toward the front). The former is required for learned vocal production throughout the life of the bird and includes the nucleus known as the HVC (this name reflects a convention in which the acronym is currently used as its proper name), a subset of whose neurons project to the robust nucleus of the arcopallium (RA). RA projection neurons, in turn, synapse directly onto brainstem motoneurons of the tracheosyringeal nucleus (McCasland 1987; Nottebohm et al. 1976). Importantly, this neuroanatomical pathway comprises a direct projection from the cortex to the motoneurons controlling muscles used for vocalization, described above as a critical feature of vocal production learners. In this case, the cortical region RA directly contacts the motoneurons that control the syrinx, or song organ.

Like the posterior vocal pathway, the anterior forebrain pathway also begins with the HVC, where a separate subset of neurons innervates the basal ganglia nucleus known as area X. Area X projection neurons synapse in the dorsolateral medial thalamus, whose neurons then project to the lateral magnocellular nucleus of the anterior nidopallium (LMAN). LMAN projection neurons join the posterior and anterior pathways via their synapses in the RA (and they also project back to area X; Bottjer et al. 1989; Okuhata & Saito 1987; Scharff & Nottebohm 1991; Sturdy et al. 2003). The anterior forebrain pathway thus forms a loop between cortex, basal ganglia, and thalamic structures, and back to cortex, and resembles cortical-basal ganglia loops in humans that are important for the initiation of movements and procedural learning (Barnes et al. 2005; Bottjer & Arnold 1997; Graybiel et al. 1994).

Given that the posterior vocal pathway controls learned vocal output, what is the importance of the anterior forebrain pathway that feeds into it? The short answer to this question is "change." Beginning in 1984 (Andalman & Fee 2009; Bottjer et al. 1984; Brainard & Doupe 2000b; Kao et al. 2005; Olveczky et al. 2005; Scharff & Nottebohm 1991; Williams & Mehta 1999) and continuing until the present, a set of elegant experiments has systematically demonstrated that the anterior forebrain pathway is required for any modifications to song, whether it be an improvement in vocal output or a deterioration. Thus, the posterior pathway can be viewed as the "command" module for learned vocal output (e.g. "sing this!"), while the anterior forebrain pathway can be seen as providing the signal for changing song, which is critical for the trial-and-error aspect of procedural learning, here in the vocal domain. As we will see below, the

anterior forebrain pathway remains important after a song is learned even in species that sing stereotyped songs such as the zebra finch.

The similarity between mammalian basal ganglia loops and song circuitry extends beyond anatomical connectivity to the identity of the cell types that make up each region, and to the neurochemicals that modulate their function. In terms of neuronal phenotypes, area X is now known to be composed of both striatal and pallidal neurons whose properties exhibit striking similarities to mammalian, including primate, basal ganglia neurons (Farries & Perkel 2002; Goldberg et al. 2010; Goldberg & Fee 2010; Reiner et al. 2004). With regard to neuromodulation, area X receives dense dopaminergic input (Bottjer 1993; Lewis et al. 1981), which modulates the excitability of medium spiny neurons via dopamine receptors (Casto & Ball 1994; Ding & Perkel 2002). Similar inputs to the mammalian striatum are critical for motor learning and reward (Balleine et al. 2009). Dopaminergic inputs to area X are differentially activated during singing, depending on the social context in which it takes place. When a male sings to a female zebra finch, his dopamine levels rise in area X and his song is more precise (Hara et al. 2007; Leblois et al. 2010; Yanagihara & Hessler 2006). When the male practices his song alone, dopamine levels are lower and songs, though still stereotyped, are more variable. Thus, social interactions modulate song circuit function by regulating dopamine release into area X, very likely during learning (Kojima & Doupe 2011), but also in maturity. These observations about the role of the basal ganglia in songbird vocal learning suggest that we should look for similar roles of the human basal ganglia in speech development, and conversely, to determine how dysfunction in this pathway impairs speech.

2.3 The KE family: A case study in disrupted vocal production learning

The first single mutation to be linked to a language disorder occurs in the gene encoding the transcription factor known as FOXP2 (Balter 2001; Fisher 2006; Lai et al. 2001). Transcription factors affect the expression of suites of other genes by binding to regulatory regions in the noncoding portion of their targets and either increasing or decreasing their transcription. The FOXP2 discovery arose from the study of a British family known as the KE family (Hurst et al. 1990), half of whom suffer from developmental dyspraxia, a deficit in the control of complex sequential movements of the orofacial muscles including those used in speaking. Peripheral control of these same muscles appears unimpaired, and innate behaviors such as suckling, chewing, and blinking are normal. These observations indicate that the problem lies within the brain rather than between motoneurons and their muscle targets – a proposition that has been confirmed by brain imaging studies.

Magnetic resonance imaging reveals that affected family members have altered amounts of gray matter relative to their unaffected counterparts in cortical and basal

ganglia regions (Belton et al. 2003; Watkins et al. 1999). These findings are consistent with the known role of other Forkhead-type transcription factors in driving embryogenesis of different organs during development. In this case, FOXP2 likely participates in the structural differentiation of brain regions. Following development, their altered structure contributes to their dysfunction. Accordingly, functional neuroimaging of the KE family reveals abnormal activation of these regions only in affected members during verbal fluency tasks (Liegeois et al. 2003). As can be imagined, the KE family has undergone extensive testing to determine the full range of their language deficits. Discussion of the complete syndrome is beyond the limits of this chapter and the interested reader is referred to Vargha-Khadem et al. (2005). It is important to acknowledge here that the phenotype is not limited to language, as affected KE family members have a significantly lower, albeit overlapping, verbal and performance IQ compared with unaffected members. In general, deficits are greater for language production than comprehension. Accordingly, assessment of core deficits, namely tasks in which affected family members' performance is poorer than and nonoverlapping with the performance of unaffected members, identified the accuracy and consistency of speech (Vargha-Khadem et al. 2005). Meanwhile, their ability to name objects is unimpaired. Thus, of the three components of language described by Hauser et al. (2002) (i.e. recursion, conceptual-intentional, and sensory-motor), sensory-motor control of speech is the most clearly affected.

In 2001, the genetic basis of the KE family disorder was shown to lie within the FOXP2 coding sequence (Lai et al. 2001). Afflicted KE family members share a point mutation on one allele for *FOXP2* that results in a substitution of amino acid 553 from an arginine to a histidine. This change occurs in the DNA binding domain of the protein, critical for its gene regulatory role. Indeed, x-ray crystallography-derived structural models of the protein show that residue 553 is intimately associated with the DNA during binding (Stroud et al. 2006). While extremely rare, individuals within other families have now been identified who exhibit strikingly similar symptoms to those described for the KE family. In these distinctive cases, disruption of the *FOXP2* gene has been consistently demonstrated (Macdermot et al. 2005; Zeesman et al. 2006). Taken together, this body of work firmly establishes that mutations restricted to the *FOXP2* gene alone can produce a profound and complex disorder of human language.

3. From gene to phenotype: How to connect them?

On the one hand, knowing how a specific genetic mutation produces a change in protein structure that results in altered brain morphology and a fully characterized language disorder would seem to form a startlingly complete picture of things. On the other, this set of observations reveals only the edges of a glimpse into the biological

basis, and thus the evolutionary origins, of language. To paint a fuller picture, the intervening molecular, cellular, and circuit effects of altered FOXP2 must be filled in. This requires carefully controlled physiological experiments using in vitro preparations and animal models (White et al. 2006). For starters, as a transcription factor, FOXP2 by itself is ineffectual and can only exert its function on brain tissues indirectly, through regulation of its target genes. Thus, we need to know what those genes are – a topic we will return to below – and how their altered levels impact language development. Given the significant parallels between songbirds and humans in vocal production learning and its underlying circuitry, which includes brain regions affected in the KE family phenotype, songbirds present a relevant animal model for exploring FoxP2. Thus, shortly after the discovery of the FOXP2 link to language, my colleagues and I examined *FoxP2* mRNA in zebra finch brains and compared the expression pattern in hatchlings with that in the human embryonic brain. We found strong expression in the basal ganglia and thalamus as well as in the cortex of both species, consistent with a role for this Forkhead transcription factor in forming these neural structures during embryonic development (Ferland et al. 2003; Haesler et al. 2004; Lai et al. 2003; Takahashi et al. 2003; Teramitsu et al. 2004). The similar expression pattern provided a "green light" to continue testing FoxP2 function in birds, with the goal of applying what we find to other vocal production learners, including humans.

3.1 Beyond brain structure: FoxP2 as a plasticity gate

In addition to its role in forming neural structures that are later used in vocal production learning, FoxP2 appears to have ongoing functions within these structures, including during learning and in the mature organism. In zebra finch song circuitry, FoxP2 expression persists into adulthood. Importantly, the adult expression is not simply a developmental vestige, but is under active regulation, as FoxP2 mRNA and protein rapidly decrease in area X of the striato-pallidum when adult birds sing (Miller et al. 2008; Teramitsu & White 2006). This "online" regulation, precisely in the striato-pallidal subregion dedicated to song and precisely when birds engage in singing, strongly implicates the molecule in the postorganizational function of this structure.

This idea is supported by the work of Haesler and colleagues, who developed a lentivirus bearing short interfering hairpin RNA (shRNA) constructs designed to knock down FoxP2 levels in the zebra finch brain. The virus was injected bilaterally into area X of 23-day-old male finches to test whether this would interfere with sensorimotor learning (Haesler et al. 2007). Control birds received injections of virus encoding an shRNA that did not target any zebra finch genes. All juveniles underwent normal tutoring, and multiple features of their song learning were assessed. Strikingly, at maturity, birds that had received the FoxP2 knock-down construct exhibited less

precise copying of their tutors' songs than did the controls. The decreased similarity included omissions, repetitions, and abnormally variable durations of syllables. This groundbreaking work represents the first case of genetic interference in songbirds resulting in documented changes to their song. Although, conceivably, altering the expression of any major transcription factor in cells that control song might result in song abnormalities, the fact that FOXP2 is vital for normal human language is consistent with the idea that the imprecise copying in FoxP2 knock-down birds reflects its specific contribution to vocal production learning.

Findings from my own laboratory complement Haesler et al.'s results. Briefly, behavioral states known to naturally lower FoxP2 in area X also give rise to more variable songs. To test this, we carefully analyzed songs of young birds that were behaviorally manipulated to achieve high vs. low levels of FoxP2 (Miller et al. 2010), using software designed to analyze zebra finch song (Tchernichovski et al. 2000). On one day, birds were allowed to sing for two hours in order to drive down levels of FoxP2 in area X, and then their subsequent songs were recorded (designated S–S, for singsing). On the next day, the same birds did not sing for two hours, which we know from our previous work leaves FoxP2 levels high in area X. The birds were then allowed to sing (designated NS-S, for nonsinging followed by singing) and those songs were recorded. S–S versus NS-S days were counterbalanced across birds to preclude any effect of order. The songs sung under each condition were then compared. The results firmly support the model, as follows: after song practice, a time coincident with low area X FoxP2 levels, vocal variability is high in both phonological (spectral features of syllables) and sequential (syllable order) domains. By contrast, when the same birds refrain from singing – coincident with high area X FoxP2 – their songs become more stable, which could reflect reinforcement of optimal motor patterns. Examples that illustrate this effect are shown in Figure 1A for spectral (phonological) features of song and Figure 1B and C for sequential features.

Together, these discoveries raise the hypothesis that FoxP2 plays a postorganizational role in vocal production learning by acting as a "plasticity gate." Behaviorally driven down-regulation of FoxP2 during song learning and adult song practice enables vocal variability. Conversely, high FoxP2 levels appear to promote organization of neural tissues during early development and may also reinforce optimal motor patterns during song learning and adult maintenance. More generally, cycles of practice and performance may improve a motor skill by altering expression levels of molecules that limit plasticity but promote reinforcement/stabilization. At first pass, this hypothesis is consistent with the lack of speech accuracy described for affected KE family members (Vargha-Khadem et al. 2005). It is important to note, however, that the KE phenotype arises from both organizational and postorganizational effects of the mutation, which is present from conception onward. Therefore, it is impossible to tease apart which of their deficits are due to abnormal development of brain structures and which are due to abnormal function of the gene throughout life.

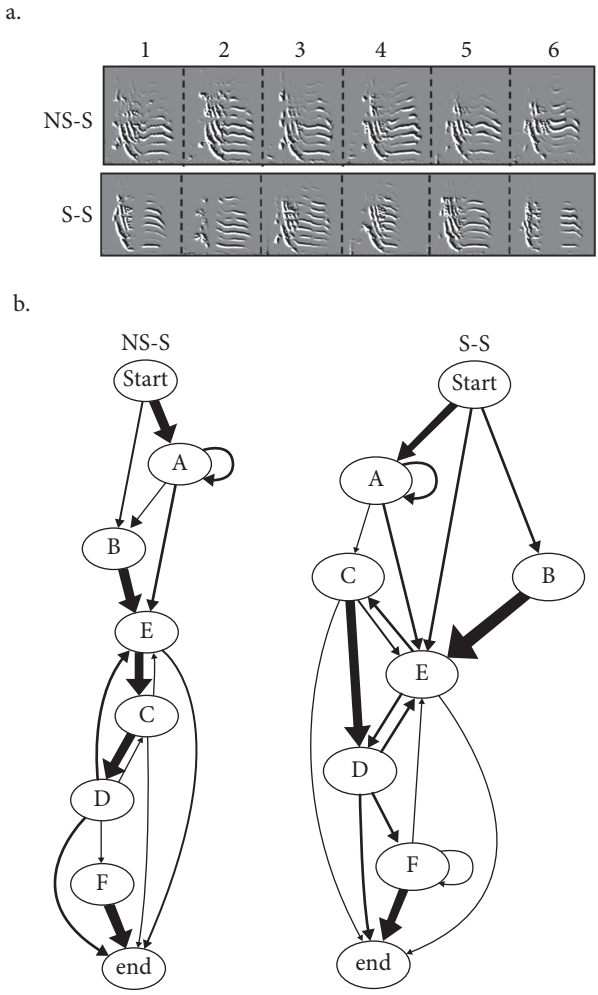

Figure 1. Phonological and sequential features are more variable under behavioral conditions known to decrease area X FoxP2 levels

A. Six renditions (1–6) of the same syllable from one bird are shown for two different days. On the first day (S–S), the bird was allowed to sing for two hours in the morning, which is known to decrease FoxP2 levels in area X. Subsequent songs contained these six syllables, which show much more variability than those shown below. This second set of syllables is from the second day (NS-S), when the bird did not sing for two hours, conditions under which FoxP2 levels remain high. Subsequently, the bird sang these more stable renditions.

B. Markov chain: an example of the possible transitions for one bird in the NS-S and S–S conditions. Letters denote syllables. Line thickness corresponds to probability; for example, in the NS-S condition, syllable E transitions 83% to syllable C (thick line), whereas a thinner line represents a 16% probability that E will end the motif; by contrast, in the S–S condition, syllable E transitions to syllable C 50% of the time, to syllable D 43% of the time, and ends the motif 7% of the time. In the NS-S condition, syllable F occurs infrequently compared to the S–S condition.

(*Continued*)

c.

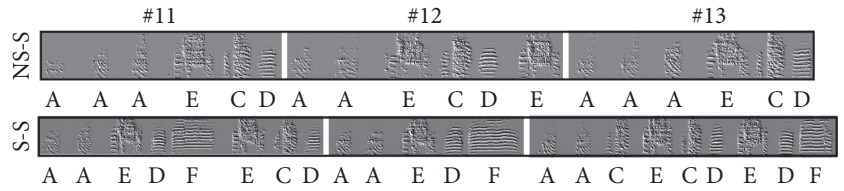

Figure 1. (Continued)
C. Exemplar of three consecutive motifs from the same bird in the NS-S and S–S conditions. Motifs occur at the same chronological order in the selected 20 motifs analyzed (#11, 12, and 13 out of 20). Individual syllables are identified by letter. In the NS-S condition, syllable A typically transitions to itself or to syllable E, and syllable C transitions most frequently to syllable D. By contrast, in the S–S condition, A also transitions to C (#13) and C to E (#13) as well. In the S–S condition, syllable F is observed (#11, 12, 13) and follows syllable D while in NS-S, syllable D transitions to E (#12) or ends the motif (#11, 13). Adapted from (Miller et al. 2010).

3.2 Other genes: FoxP family members

The plasticity gate hypothesis for FoxP2 arose largely through songbird research and remains untested in humans. Indeed, only if and when we are able to perform *FOXP2* gene therapy in humans would such a test be possible, due, in part, to the issue raised above about distinguishing organizational from postorganizational effects. A separate hypothesis arising from songbird research, however, has now been confirmed for human language. Collaborative work between my laboratory and that of Dr. Daniel Geschwind (UCLA) revealed that *FoxP1* and *FoxP2* share remarkably similar expression patterns in human and zebra finch brains (Teramitsu et al. 2004). Our observation that *FoxP1* is expressed in a sexually dimorphic pattern within zebra finch song circuitry led us to hypothesize that, like FOXP2, FOXP1 plays a role in vocal production learning and could underlie language-related disorders. Remarkably, this prediction has been borne out through the discovery of multiple human cases in which *FOXP1* mutations are associated with language deficits, accompanied by more global changes in cognitive abilities (Carr et al. 2010; Hamdan et al. 2010; Horn et al. 2010; Pariani et al. 2009). In several of these cases, the only gene shown to be disrupted is *FOXP1*, pinpointing it as an additional molecule critical for normal language development.

3.3 Genes downstream of FOXP2

Since FOXP2 is a transcription factor, its role in speech and language must be mediated by regulation of its target genes. Thus, we and others have hypothesized that *FOXP2* is not "*the* gene" for language, but rather represents an entry point into a network of molecules important for language (reviewed in Fisher & Marcus 2006;

Hilliard & White 2009). Finding downstream targets and identifying their function promises to elucidate the neuromolecular basis of language and disorders in which language is affected, such as specific language impairment (SLI) and autism spectrum disorder (ASD). Several exciting approaches have been taken to identify FOXP2 gene targets, focusing on those in humans. Two studies utilized a technique known as ChIP-chip – for chromatin immunoprecipitation followed by arraying on a microchip – that assures that identified genes are directly regulated by FOXP2. In both, an antibody against FOXP2 protein was used to specifically detect and isolate FOXP2 while doing its job of regulating transcription, that is, while FOXP2 was bound to the DNA regulatory sequences in the promoters of its target genes. These targets were then identified using promoter microarrays. In one study, human fetal lung, inferior frontal cortex, and basal ganglia tissues were used to identify target genes (Spiteri et al. 2007), with eight co-occurring in the two brain areas, but not in the lung. The different suites of genes regulated by FOXP2 depending upon the tissue help to explain the brain-specific functions of FOXP2. In the other study, human neuronal-like cell lines were similarly tested and revealed 119 targets (Vernes et al. 2007), with significant overlap with those identified in the former work. These studies do not represent a complete list of FOXP2 neural targets – not all neuronal cell types nor all known promoters were available. Such limitations will undoubtedly decrease with technological advances, promising a more complete picture of human FOXP2 targets.

Since humans are uniquely capable of language, which FOXP2 targets are uniquely human becomes of interest. The above studies used human tissues but did not show whether these same targets would also be regulated by FOXP2, for example, in our closest relative, the chimpanzee. To address this question, two additional studies have identified genes whose expression is altered specifically by the protein form of FOXP2 that exists in humans. In one, human neural progenitor cells were transduced to produce either the chimpanzee or the human FOXP2 (Konopka et al. 2009) and subsequent changes in gene expression were compared. The authors found 61 genes that were significantly upregulated and 55 genes downregulated in cells transduced with the human FOXP2 compared to those transduced with the chimp form.

Neither chimps nor zebra finches are easily amenable to transgenic approaches for altering gene expression, whereas mice are. Thus, a separate study introduced the human *FOXP2* into the endogenous form found in mice and examined the resultant changes in neuronal gene expression (Enard et al. 2009). The authors identified 34 genes whose expression differed specifically within the striatal region of the mouse basal ganglia. Medium spiny neurons are the main cell type in this area, and their dendrites – the neuronal processes upon which they receive synapses – were longer in the mice expressing the human form of FOXP2, suggesting the potential for enhanced neuronal "cross talk." In line with this, a form of synaptic plasticity thought to underlie certain forms of motor skill learning in mice was enhanced in this region. This

finding is remarkable because it directly complements a prior study in which, rather than inserting the normal human form of *FOXP2* into mice, the mice were mutagenized such that they possessed the KE family form of *FOXP2* (Groszer et al. 2008; Teramitsu & White 2008). In contrast to enhanced striatal plasticity, these mice were deficient in the very same form of synaptic change. Additionally, they exhibited deficits on the accelerating rotarod, a form of motor skill learning thought to be supported by this very type of synaptic plasticity.

What about their vocalizations? While the long answer may eventually be forthcoming, the short answer is that the type of vocalization tested thus far in all of the mice described above is an unlearned one, namely the ultrasonic cries of mouse pups when isolated from their mothers. Since mice are not capable of hearing until their second postnatal week (Ehret 1976), any alteration in these isolation calls reflects a change to an innate vocalization. As mice mature, they produce additional ultrasonic vocalizations that have even been likened to birdsong (Holy & Guo 2005). To what extent these "mouse songs" require learning is an exciting new area of intense investigation, and the experiments are following the general design discussed above for testing vocal learning in any species. At the time of writing, a first report has been published in which young mice of one strain were exposed to mature mice of a separate one, reminiscent of the developmental experience of the young African elephant who was housed with Asian elephants. In this study, normal mouse pups from one strain did not learn the songs of their foster-parents (Kikusui et al. 2011). Thus, there is as yet no murine equivalent of Calimero. As more of the mice with altered *FOXP2* genotypes reach maturity and become available for testing, new findings about their vocal output will be forthcoming. Likewise, follow-up experiments are required for the genes outlined above whose expression is altered depending on the FOXP2 isoform. Such work has begun for one of these gene targets, known as contactin-associated protein like-2 (*CNTNAP2*), and has already yielded important information about how FOXP2 connects to language uniquely in humans.

3.4 Key detour? A FOXP2 target is linked to specific language impairment and autism spectrum disorder

While FOXP2 has been linked to language in multiple cases, evidence for its role in SLI or in other developmental disorders in which language is affected, such as ASD, has been lacking (cf. Li et al. 2005; but see Peter et al. 2011). Yet, as described above, FOXP2 regulates many genes, the exact identities of which depend upon the tissue. Excitingly, a series of studies have now shown that *CNTNAP2* is implicated in developmental disorders of language, and is a direct target of FOXP2 repression in humans (Vernes et al. 2008; Whitehouse et al. 2011). This discovery arose, in part, through a modified version of the ChIP-chip technique described above. In this variant, known as ChIP-seq,

the gene targets to which FOXP2 binds are directly sequenced, rather than arrayed. One of the sequenced genes encodes CNTNAP2 (also referred to as AUTS15, CASPR2, CDFE, DKFZp781D1846, and NRXN4), a member of the neurexin superfamily of cell-adhesion molecules that, together with their binding partners, the neuroligins, have been implicated in ASD (Poliak et al. 1999). Several independent lines of evidence have converged to identify CNTNAP2 as an important modulator of diverse clinical phenotypes involving impaired language performance. *CNTNAP2* was originally linked to SLI and ASD in an Old Order Amish population that harbored an abnormal *CNTNAP2* allele. A single nucleotide deletion resulted in a frame shift and premature stop codon, producing a truncated protein that lacks its transmembrane and intracellular domains. This truncation presumably disrupts the protein's normal function. Members of the population homozygous for the mutation exhibit cortical dysplasia-focal epilepsy and symptoms of ASD and SLI (Strauss et al. 2006).

Though the truncated *CNTNAP2* described above results in a severe phenotype, less dramatic polymorphisms in the general public have been linked to ASD and SLI, using instruments of autism diagnosis, age of first word, language expression and comprehension, ability to repeat nonsense words, and reading ability (Alarcon et al. 2008; Arking et al. 2008; Newbury et al. 2010; Vernes et al. 2008). Most recently, common *CNTNAP2* variants have been shown to influence early language development even among the general population (Whitehouse et al. 2011). Since *CNTNAP2* is expressed in neurons and is associated with cognitive disorders, several groups have looked for anatomical anomalies in the brain associated with *CNTNAP2* polymorphisms. Structural MRI of affected members of the Old Order Amish revealed abnormalities in the temporal lobe and striatum, areas critical for speech and language (Strauss et al. 2006). In a separate study outside of that population, people homozygous for a risk allele of *CNTNAP2* had less white and gray matter than those bearing nonrisk alleles in several brain regions associated with ASD (Tan et al. 2010). Functional MRI has revealed altered frontal lobe connectivity associated with *CNTNAP2* risk alleles (Scott-Van Zeeland et al. 2010). Curiously, the neuroanatomical changes in humans are not mimicked in *Cntnap2* knockout mice, which exhibit typical brain morphology. In fact, pending further characterization, knockout mice display surprisingly normal anatomical, neurophysiological, and behavioral phenotypes (Poliak et al. 2003).

The difference between human and rodent Cntnap2 phenotypes may be a function of where Cntnap2 is expressed in the brain of each species. In human fetal brains, prior to myelination, *CNTNAP2* is highly enriched in the frontal cortex and otherwise restricted to the striatum and dorsal thalamus, defining key circuitry important to aspects of higher cognition, including the implicit learning essential for language development (Abrahams et al. 2007). This stands in sharp contrast to the broad transcript distribution observed in the developing brains of both rats and mice. While the jury is still out on the degree to which rodent vocalizations are learned, it is clear

that birdsong is. Intriguingly, we found that the *Cntnap2* expression pattern in zebra finch brains is more similar to the human pattern, which is not exhibited by rodents (Panaitof et al. 2010). *Cntnap2* mRNA is differentially expressed in several parts of the song circuit, including enrichment in the RA and LMAN cortical regions, relative to the surrounding areas that nonetheless contain similar cell types. In the basal ganglia song nucleus area X, there is a marked reduction in *Cntnap2* mRNA, relative to its surrounding region. Taken together, these findings support the hypothesis that CNTNAP2 plays an early developmental role in the patterning and functional specialization of circuits related to higher cognition and learned vocalizations, potentially in multiple species.

3.5 Looking into the dark matter

The demonstration of an interaction between FOXP2 and CNTNAP2 in humans (Vernes et al. 2008) begins to define a neuromolecular network related to language and could underlie learned vocal communication in other species. As detailed above, within a given species, FOXP2 interacts with different suites of genes in different tissues, which helps to explain how the effects of its mutation are largely restricted to the brain. Further, FoxP2 likely interacts with different suites of genes in the same tissue of different animals, as evidenced by the work comparing differential gene regulation due to human versus chimpanzee forms of FoxP2. This phenomenon can provide hints to the biological origin of the language phenotype. For example, in the case of CNTNAP2, the genetic region to which FOXP2 binds and represses transcription is located in an intron. Introns, like promoters, are noncoding regions of the DNA and can contain regulatory sequences that indicate where, when, and how much of the gene will be expressed. Two consensus sites for FOXP2 binding were found within this intronic region, namely two instances of the DNA sequence CAAATT (Vernes et al. 2008). If these sites are lacking in the *Cntnap2* of rodents, then Foxp2 may be unable to repress rodent Cntnap2 expression. This possibility fits with the restricted pattern of *CNTNAP2* expression observed in human fetal brains, which is inverse to *FOXP2* expression therein, and contrasts with the diffuse pattern of *Cntnap2* expression observed in mice and rats (Abrahams et al. 2007). Specific repression of *CNTNAP2* during human brain development could thereby enhance the functional connectivity of brain areas critical for language development (Scott-Van Zeeland et al. 2010).

Whether or not the FOXP2-CNTNAP2 connection in humans represents a key evolutionary detour, it serves to illustrate a broader point, namely that regulatory sequences in the noncoding regions of genes, in the so-called "dark matter," are important players in evolution. The human genome project has revealed that 44% of our DNA is composed of mobile transposable elements (Lander et al. 2001). It has

been speculated that short regulatory regions such as transcription factor binding sites can be present in these mobile elements and thereby produce species-specific gene expression patterns (Britten & Kohne 1968). Strong support for this scenario has recently been provided through study of the gene encoding human cathelicidin antimicrobial peptide (CAMP; Gombart et al. 2009). Specifically, the binding site for the vitamin D receptor was shown to be present in the promoter for this gene in the primate lineage, including humans, but not conserved in nonprimate mammals. Insertion of this site was mediated by a primate-specific Alu family of mobile, middle repetitive short-interspersed elements. As a result of this added regulatory region, vitamin D is able to potentiate the innate immune response in human and nonhuman primates but does not do so in other mammals.

Much attention has been paid to interspecies variation in the coding sequences of FoxP2, with the important finding that, among primates, two amino acids are unique to humans (Enard et al. 2002). Again, this discovery enabled the identification of certain genes that are uniquely regulated by the human, as opposed to the chimpanzee or mouse, form of FoxP2 (Konopka et al. 2009). In addition to coding sequences, however, alterations in the noncoding "dark matter" can give rise to important species-specific changes. Such changes are not limited to what has been uniquely added in humans, as just described for the CAMP gene (Gombart et al. 2009) and speculated on for CNTNAP2, but also to what is uniquely lacking. A recent report now demonstrates that, relative to the chimpanzee, ~500 genes have undergone human-specific deletions which are largely restricted to their noncoding regions (McLean et al. 2011). Perhaps not surprisingly, both *FOXP2* and *CNTNAP2* are on this list. To validate the potential impact of these changes, the authors demonstrated that one of the deletions removes a regulatory region of a growth arrest gene and is correlated with the expansion of specific brain regions in humans. In this way, deletions or insertions of even short pieces of DNA that happen to contain consensus sites for transcription factor binding can shape human evolutionary divergence.

3.6 Follow-through: Prioritizing genes

Given the startling amount of genomic complexity, how can we prioritize genes and gene interactions for investigation into language origins? Clearly, multiple approaches are needed, as in the case of CNTNAP2, where, despite being one of many FOXP2 targets uncovered in the ChIP-seq experiment, independent lines of converging evidence for its association with language brought it to the forefront (Vernes et al. 2008). To go beyond "one gene at a time," statistical techniques for probing correlations in gene expression are being generated and used to highlight gene interactions that are unique to brain regions that support specialized human cognitive capacities (e.g. Oldham et al. 2006). One of these techniques, known as weighted gene coexpression

network analysis (WGCNA) is at the forefront of modern tools required to analyze high dimensional data sets while avoiding the pitfalls of multiple hypothesis testing (Zhang & Horvath 2005). The approach highlights clusters of genes whose expression levels change in concert, and groups them into modules, with genes at the center of the modules being the most highly correlated, or connected – so-called "hub" genes. This methodology has an outstanding track record in predicting novel genes within highlighted pathways, such as previously unknown molecular targets in cancer (Horvath et al. 2006). It has even been fruitfully applied to clustering voxels (WVCNA), rather than genes, in fMRI data (Mumford et al. 2010).

One study has applied WGCNA to gene expression data from human fetal brains (Johnson et al. 2009). The analysis highlighted 11 hub genes as being critical for human brain development. Excitingly, four of these exact molecules have recently been shown to have undergone the human-specific loss of their regulatory DNA (McLean et al. 2011), relative to chimpanzees, while five are in the same family as genes that bear the human-specific deletions. This is another instance of the highly predictive value of WGCNA. I and my colleagues have applied this approach, for the first time, to a procedurally learned behavior, by examining suites of genes that are coregulated in songbirds during singing. We used the same paradigm described above in which birds alter their own area X FoxP2 levels as a function of how much they sing (Hilliard et al. 2012) in order to highlight genes that are coregulated with and functionally interact with FoxP2. Comparison of these to the known targets in humans reveals shared evolutionary drivers of vocal production learning, as well as molecular interactions unique to humans. The latter represent high priorities for further investigation as to their role in language origins.

4. Summary

This chapter has focused on one subcomponent of language, namely vocal production learning. We have argued that probing the neural circuitry that gives rise to this behavior, as well as what happens in cases where it malfunctions, can highlight the relevant biology upon which evolution has acted. Much progress has been made in understanding how the brain accomplishes this sensorimotor feat in the vocal domain by using a songbird animal model that is "expert" in this capacity. While birdsong and speech evolved independently, the brain appears to have found similar biological solutions to the challenge of learning to communicate vocally. Other animals offer distinct insights for other language subcomponents (cf. Zuberbühler, this volume). In all of these domains, analysis of gene interactions, largely mediated through the noncoding regions of the genome, provide even more biological fodder for evolutionary change.

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